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in Pathological States: Chemical and Biochemical Studies of PGB$_x$

by

Thomas M. Devlin, Ph.D.

Department of Biological Chemistry
Hahnemann Medical College and Hospital

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PGB$_x$, a derivative of 15-keto prostaglandin B$_1$, protects oxidative phosphorylation in isolated mitochondria and tissues during ischemic insult. These unique activities suggest a potential therapeutic role for PGB$_x$. The principal components of PGB$_x$ samples are oligomers containing 4 to 8 monomers of the 15-keto PGB$_1$, but impurities formed during the hydroxide catalyzed oligomerization process are also present and difficult to remove.

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This program is responsible for the synthesis and purification of PGB_x, testing samples, and studies of its in vitro and in vivo activity. Different procedures, including gel filtration, electro-focusing and chromatography have been evaluated to purify PGB_x prepared in this laboratory and to determine if a separation of the different biological activities of the stock material can be achieved. C^{13}-NMR studies indicate that PGB_x is a simple oligomer with bonding between the chains.

Purified PGB_x samples and analogues supplied by other investigators are evaluated for possible protective effects on oxidative phosphorylation. PGB_x interacts with a specific protein of the mitochondrial F_1F_0-ATPase, the principle complex involved in energy transduction. PGB_x is a Ca^{2+} ionophore with both natural and synthetic membranes and is nearly as effective as the best known bacterial ionophores. The material has an effect on the release of Ca^{2+} from mitochondria unrelated to its ionophoretic activity; results suggest a control on intramitochondrial Ca^{2+} levels. Studies of the quenching of the intrinsic fluorescence of PGB_x by divalent cations have yielded information concerning its mode of action as an ionophore.

PGB_x protects isolated rat hearts from damage during periods of hypoxia. Hearts perfused with an oxygenated medium will beat for several hours but cessation of perfusion leads to permanent loss of mechanical activity; PGB_x administration protects the heart allowing recovery of activity. PGB_x also protects the rat against the development of a stress ulcer in the stomach, another example of a protection against tissue ischemia. Pretreatment with PGB_x increases survival time and cardiac activity of mice placed in low oxygen tensions. These results demonstrate the unique pharmacological properties of PGB_x and suggest its value in protecting tissues against hypoxia.

DEPARTMENT OF BIOLOGICAL CHEMISTRY
HAHNEMANN MEDICAL COLLEGE

I. Introduction

As in last year's Annual Report, this report will be a composite of the
activities carried out by the Department of Biological Chemistry at the
Hahnemann Medical College and at the Naval Air Development Center (NADC),
Warminster, Pennsylvania. In 1979, two existing contracts, one to each of
the above mentioned groups, were combined into one contract awarded to the
Hahnemann Medical College and Hospital. The combined contract led to a more
integrated research activity and an increase in professional staff interaction,
even though the two groups are physically separated and each has individual
goals and objectives. The activities of the two groups are clearly delinated
and are presented separately in this report.

In addition to the activities at the NADC supported by this contract, Dr.
H. Shmukler directs a separately funded activity in the same laboratories.
The two NADC groups function as an integrated total unit and thus the report
from the NADC presented here overlaps with the reports submitted separately by
Dr. Shmukler. This is considered appropriate in that it was deemed advisable
to prepare a comprehensive report for the activity at NADC.

Both the Hahnemann and NADC units perform a service function. It is the
responsibility of the Hahnemann group to carry out assays of samples submitted
by other contractors who are conducting purification studies of PGB_x or
preparing analogues. The NADC group is the principle laboratory for the large
scale purification of intermediates for the synthesis of PGB_x and for the actual
polymerization of the 15-keto PGB_1 into PGB_x. The service activities of this
group are coordinated with the contract awarded to Dr. George Nelson of the
Department of Chemistry, St. Joseph's University, who has been responsible
for the synthesis of the intermediates required for PGB_x synthesis. The NADC

group is also responsible for purification and stock piling of standard PGB_x and various intermediates and for supplying samples on request to other investigators.

II. Objectives

The long range objective of the program is to gain an understanding of the events leading to the loss of membrane energy transduction mechanism, particularly oxidative phosphorylation and ion transport, in pathophysiological conditions such as anoxia as a means to develop approaches to protect tissues or cells from such insults. The objective for 1981-1982 is to continue the study of the activity of PGB_x and its analogues on both isolated mitochondria and in intact tissues with the goal to determine if PGB_x or one of its analogues could be useful as a therapeutic agent in conditions where tissue anoxia leads to the loss of mitochondrial oxidative phosphorylation. As part of these studies this program will perform a support role to test the activity of purified samples of PGB_x and analogues of PGB_x such as the oligomers of the ethyl-analogue. The contractor will continue to synthesize and purify PGB_x and will conduct physical chemical studies of PGB_x directed to determining the structure.

A. Specific Goals of the Hahnemann Research Group: (1) To employ the in vitro assay systems developed and refined in 1979-1980 for evaluation of PGB_x samples as a guide to isolation and identification of the active component, to evaluate samples of analogues of PGB_x for their ability to protect oxidative phosphorylation and to develop additional in vitro systems including assays for ionophoretic activity to measure PGB_x activity. (2) To continue an investigation of the effect of PGB_x on mitochondrial oxidative phosphorylation as one direction in determining the mechanism of PGB_x protection of aged mitochondria. (3) To continue the evaluation of ionophoretic properties of PGB_x and its

analogues both in mammalian and artifical membrane systems to determine if this activity is the explanation for some of the manisfestation in vivo of PGB_x.
(4) To continue development of the perfused heart system as well as other intact tissue systems for studying the effect of PGB_x and its analogues in protecting the tissue from periods of anoxia.

B. <u>Specific Goals of the NADC Research Group</u>: (1) To complete purification and stock piling of 15-keto B_1 from precursors supplied by Dr. G. Nelson, St. Joseph's University. (2) To complete the synthesis of PGB_x from stocks of 15-keto PGB_1. (3) To continue studies on the purification of PGB_x to increase the stock of the PGB_x Type II and to stockpile unique purified fractions of PGB_x for evaluation of biological activity. (4) Attempt to separate the PGB_x protective activity from the inhibitor activity. (5) Continue physical chemical studies directed towards determination of the chemical structure of PGB_x. (6) To continue studies of the in vitro effect of PGB_x and to evaluate PGB_x in selected animal model systems.

III. <u>Report of the Research Program at Hahnemann</u>

A. <u>Background</u>: A detailed report of the earlier studies concerning the activity of PGB_x have appeared in previous Annual Reports. The following highlights only the major activities of this laboratory in previous years and brings these results into focus with the basic problem and objectives of the program. Studies in this laboratory have confirmed and extended the observations of Polis, Polis and Kwong (Proc. Nat. Aca. Sci., USA, (1979), <u>76</u>, 1958), that a polymeric form of 15-keto prostaglandin B_1 (designated PGB_x) is capable of protecting oxidative phosphorylation of isolated rat liver mitochondria which had been aged at 0° for several days. These observations have been extended to demonstrate that PGB_x will protect freshly isolated mitochondria from changes in the energy transduction for short periods at 30^0 which greatly simplifies the investigation

in that it reduces the vagaries of the aged mitochondrial assay system. One difficulty is that PGB_x samples also have an inhibitory effect on oxidative phosphorylation at concentrations higher than that required for protection.

The complexity of the energy transduction mechanism of mitochondria has been reviewed in previous reports as well as the sensitivity of this system to a variety of treatments, chemical agents and pertubations of the membrane. It is very difficult to single out a specific chemical event which PGB_x might be either preventing or inhibiting. A variety of events could occur during the aging process or during incubation at 30°, any of which could be prevented by PGB_x. In fact, as indicated below, PGB_x has several different in vitro effects on membranes and there is some indication that its effect on the 5 day aged preparation and that on incubated freshly isolated mitochondria may be different. In can be concluded, however, that PGB_x has no ability to reverse already damaged mitochondria but rather has its effect by preventing some deleterious activity from occurring. Previous reports presented evidence that PGB_x has an effect on an intermediate in the energy transduction mechanism, specifically one of the components of the F_1F_0-ATPase. These observations, however, do not exclude the possibility of a direct effect on some other pathway or activity of the mitochondria. As will be discussed in the results section, there is evidence that PGB_x may have direct effects on the mechanisms for transmembrane movement of divalent cations particularly Ca^{2+}.

In 1978, we demonstrated (Ohnishi, S.T. and Devlin, T.M., Biochem. Biophys. Res. Comm., (1979), 89, 240) that PGB_x has ionophoretic activity capable of translocating Ca^{2+} as well as other divalent cations across biological membranes. Our evidence clearly supports the proposal that PGB_x serves as a true ionophore and not creating a channel in membranes. The ionophoretic properties of PGB_x have been confirmed by Weisman, et al. The ionophoretic properties can be demonstrated in both naturally occurring membranes such as the plasma membrane,

sarcoplasmic reticulum and mitochondrial membranes, or in synthetic phospholipid membrane systems, i.e. liposomes. The use of liposome preparations in which a Ca^{2+} indicator is encapsulated have made it possible to quantitate the ionophoretic properties of PGB_x and to make comparisons with other ionophores and to compare samples of PGB_x and new analogues. The relationship between the ionophoretic activity and the protective action on oxidative phosphorylation of PGB_x, however, has still to be delineated; studies have been conducted in order to demonstrate a possible relationship or lack of relationship but the results are inconclusive. A major concern is whether the two activities are due to the same or different substances in samples of PGB_x. For this reason, various samples and analogues have been tested in a battery of tests to compare effects on oxidative phosphorylation and for ionophoretic activity.

The ability of PGB_x to protect oxidative phosphorylation is an extremely unusual property in that very few other compounds have been found to have any effect regardless of how minimal in the assay systems. Addition of some cofactors and bovine serum albumin, have some minor protective effect but none equal to PGB_x; in many cases their effect can be readily explained by either the loss of the cofactor or the protection from uncoupling by such agents as free fatty acids as is the case for albumin. It is concluded that the effect of PGB_x is not a property of prostaglandins and reinforces the idea that PGB_x is not functioning as a unique prostaglandin but rather that it has unique properties totally different from those observed for PGE or PGF. These in vitro results coupled with the pharmacological effects of PGB_x in protecting ischemic tissues from damage provide the motivation for the continued evaluation of the activity of PGB_x. It has been proposed that part of the irreversible damage that occurs in tissue ischemia may be the loss of the energy transduction activity of mitochondria and, thus, there is a rationale for both understanding the mechanism

of action of PGB_x at the mitochondrial level as well as to understand and describe its pharmacological activities. One anticipated result of the in vitro studies is the development of a reproducible and convenient system for evaluating any compound which might have an activity similar to that of PGB_x.

During the last two years a major effort has been undertaken by several laboratories to purify PGB_x and to prepare analogues of the material. Samples of purified PGB_x, various analogue derivatives and any sample submitted have been tested in this laboratory to determine if they have an activity similar to PGB_x. At this time, four different assay procedures are available and are utilized in evaluation of samples; the selection of the specific assay depends upon the specific needs of the supplier of the sample. As reported above, PGB_x has a biphasic effect on mitochondria causing a protection of oxidative phosphorylation at low concentrations but an inhibition at higher concentrations. A number of samples have been tested which have lower inhibitory activity but usually have lower activity in protecting oxidative phosphorylation. Some purified PGB_x samples and some analogue samples have a higher protective activity on the basis of weight but, in most cases, these samples also have a higher inhibitory activity. A few samples have been tested with significantly lower inhibitory activity but there is still not sufficient evidence to clearly eliminate the possibility that a single component is having a dual effect. Obviously, if two different substances are present in samples, one with protective action and the other an inhibitor, it should be possible to separate these two activities. It is worth noting that the relatively low level of cytocoxicity in animals of PGB_x suggests that the inhibitory activity in vitro may not be of significant consequence in vivo.

In the summer of 1980, we observed that PGB_x could protect isolated perfused rat hearts from damage during a period of anoxia. The assay procedure involved measurement of the mechanical activity which ceases when the perfusion is stopped. With reinstitution of perfusion after short time periods

(10 to 30 minutes) the heart resumes its activity. PGB_x in the perfusion medium allows the heart to remain in the anoxic state for longer periods. A major effort is underway to refine this system as a potential whole tissue assay procedure for PGB_x samples, and as a means to evaluate the biochemical events during the anoxic period causing the irreversible damage to the tissue.

B. Progress Report: This report covers the period September 1, 1980 through August 30, 1981. In some areas details are presented concerning the results but for some preliminary studies only a summary of the results are presented. Much of this work has been presented in abstract form for presentation at national and international meetings and manuscripts are being prepared for submission to major journals.

(1) Assay of Samples: During the past year, over 150 samples have been tested in the various in vitro assay systems for comparison of their activity to that of a standard PGB_x sample. Each sample was usually tested with several different mitochondrial preparations and at a number of different concentrations depending upon the request of the supplier. Samples were received and reports of results were submitted to Dr. G. Nelson (St. Joseph's University), Dr. R. Doskotch (Ohio State University), Dr. K. Biemann (MIT), Dr. D. Trainor (Columbia University) and Dr. H. Shmukler (NADC). This report does not contain detailed results on these samples; results of the assays should be reported in the progress report from the institution submitting samples to the laboratory.

Four different assay procedures, each with a different rationale, are carried out. (a) Samples are tested for protection of phosphorylating capacity of mitochondria isolated from rat liver and aged at 0° for five to eight days (aged mitochondria assay). In this assay, PGB_x is added to the mitochondria during a brief pre-incubation at the end of the aging period and the preparation tested for its capacity to catalyze phosphorylation of ADP. (b) A second assay for protection of phosphorylation involves the incubation of freshly isolated

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mitochondria at 30° for 45 minutes (pre-incubated mitochondria assay); during the pre-incubation there is a significant loss of phosphorylating capacity. ATP prevents the loss of activity during this short time period and is employed as a control for comparison to PGB_X activity. This assay procedure was described in the Annual Report of 1980. (c) The effect of PGB_X on freshly isolated mitochondria not pre-incubated is also carried out in order to test the inhibitory or uncoupling activity without concern for the protective effect. As described in 1980, there is a definite breakpoint in concentration above which there is inhibition but below which there is no inhibition at all. Below the inhibitory concentration, PGB_X will protect aged mitochondria or pre-incubated mitochondria. (d) Samples have also been tested for ionophoretic activity using isolated liposomes into which arsenazo-III has been incorporated. This compound is a sensitive indicator for Ca^{2+} and is used to measure transmembrane Ca^{2+} movements in the presence of ionophores. A number of studies have been carried out to evaluate the two systems for protection of mitochondria and there appears to be some differences. If there is any question concerning the activity of a sample, the material is tested in both the aged mitochondria and the pre-incubated mitochondria assay.

Samples prepared by Dr. G. Nelson of the ethyl analogue of PGB_X and oligomers prepared by careful control of the polymerization process have been assayed; there is evidence of protective activity in the samples but in most cases they still contain the inhibitory material. Samples from Ohio State University have on occasion demonstrated significantly higher specific activity in protecting mitochondria but a concomittant increase in the inhibitory activity. The higher specific activity would suggest that it is possible to separate out either inactive material or less active material from samples of PGB_X. Dr. Trainor has submitted several samples which she considered might be free of the inhibitory activity.

In no case has her samples been found to be significantly more active in protecting mitochondria and in most cases only slightly less inhibitory.

Some of the samples have been tested for their ionophoretic properties; the results indicate that the active ones in the phosphorylating systems contain this activity. In testing over several hundred samples during the last two years, the results would suggest that the three activities, i.e. protection of phosphorylation, inhibition of phosphorylation and ionophoretic properties may all be contained in a single compound. Final decision concerning whether these activities can be separated will depend upon the eventual purification and identification of a single compound.

During the past year, there has developed a very close working relationship between the Hahnemann laboratory and Dr. Nelson. Samples received from him are frequently tested within five working days and the results reported promptly. The biological assay systems do have inherent problems of inconsistancy depending on the mitochondrial preparations. The basic assays work most of the time but occasionally preparations and therefore the assays must be dropped because of inactive mitochondria. This is inherent in studies of a sensitive membrane system such as energy transduction. Another quirk to the system is the difference in the activities in the aged preparations versus the pre-incubation assay system. PGB_x in both cases will protect from the loss of phosphorylating capacity but recent results suggest that there might be subtle differences between the effects of PGB_x on these two different types of mitochondrial preparations. It has been demonstrated that bovine serum albumin will prevent the effect of PGB_x on the fresh aged preparation presumably because of the tight binding of PGB_x, and thus a diminution in available PGB_x in the system. On the other hand, bovine serum albumin is important to measure protection in aged mitochondrial preparations and significantly improves the effect of PGB_x. There does not seem to be the prevention of the PGB_x activity in this latter assay as observed with

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the pre-incubated mitochondria. It is interesting to note that a somewhat puzzling effect of albumin has also been observed on the ionophoretic properties of PGB_x depending on the assay. We have considered the possibility that albumin may not totally shield the active site on PGB_x and thus permit PGB_x to function in some systems but not in others.

Another difference between the aged mitochondrial preparations and the pre-incubated fresh mitochondrial preparations is the effect of Ca^{2+} chelators (EGTA). There is no effect of EGTA in protecting mitochondria which have been aged for several days at 0° but the chelator potentiates the PGB_x effect with the fresh preparations pre-incubated at 30°. The latter effect would suggest that in the pre-incubated system, Ca^{2+} is involved in the loss of phosphorylating capacity; endogenous Ca^{2+} released from the mitochondria could be sequestered by the chelator or PGB_x. Since EGTA has no effect on the aged preparation, it appears that Ca^{2+} in this case is not the principle factor causing loss of phosphorylation. Thus, in two different preparations, PGB_x is protecting but possibly by different mechanisms. It could be that the low concentration of endogenous Ca^{2+} could be sequestered by the PGB_x and, therefore, prevent Ca^{2+} from activating the mitochondrial phospholipase which could alter the mitochondrial membrane.

It has been reported by Polis, et al. (Op. Cit.) that uncoupling by 2,4-dinitrophenol at low concentrations can be prevented by PGB_x. We have not been able to consistantly repeat this observation with fresh mitochondrial preparations. On occasion, we have seen a slight protection by PGB_x but not routinely. The uncoupling activity of 2,4-dinitrophenol is complex in that it has been reported to function as a weak acid dissipating the proton gradient established during active respiration and to interact specifically with one of the components of the phosphorylating mechanism. It may be that we have not been able to establish the correct conditions for demonstrating the effect of PGB_x on the uncouplers activity. It should be noted that in a previous report we indicated that with

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fresh mitochondrial preparations dinitrophenol stimulated respiration is inhibited by PGB_X.

Another difficulty with the assay system has been the inability to routinely demonstrate an effect of PGB_X with a variety of oxidizable substrates; α-ketoglutarate is the principle oxidizable substrate in our assays. We have demonstrated protection of phosphorylation with pre-incubated mitochondria with substrates such as succinate, β-hydroxybutarate and malate + glutamate but positive results are not always observed. Again, there is no ready explanation for this lack of consistancy. Obviously we have considered possible ancillary reactions in which α-ketoglutarate may be involved and could have a role in the protective effect of PGB_X.

The studies conducted on the basic assay system demonstrate both the complexity of the problems of energy transduction and the formation of ATP and those of demonstrating the specific sites of PGB_X action. The results support the need to gain an understanding of the PGB_X effect which will not only help us in understanding the mechanism of energy transduction but should also be valuable in leading to methods to protect tissues from loss of this activity during tissue insult.

These studies required the time of one technician.

(2) Ionophoretic Activity of PGB_x

An unexplainable observation in our early studies on the ionophoretic prop-
erties of PGB_x with isolated mitochondria from various tissues was the fact that
there appeared to be a great variation in the ability of PGB_x to stimulate a re-
lease of Ca^{2+} from preloaded mitochondira. PGB_x would permit the rapid release of
Ca^{2+} from rat heart but not from rat liver. It has now been found that the ionophor-
etic properties of PGB_x with isolated mitochondria are more complex than that ob-
served with phospholipid liposomes. In the presence of acetate as a permeant ion,
PGB_x induces a loss of Ca^{2+} from preloaded rat liver mitochondria, as shown in Fig-
ure 1. Separate studies indicate that there was no major swelling of the mitoch-
ondria during the time course of the study; this was also supported by the observ-
ation that the membrane potential, measured with the dye saphranine O, decreased
by only 10 mV and did not fall below 80 mV, a value required for the thermodynamic
equilibration of the Ca^{2+} uniporter for uptake. The results demonstrate that PGB_x
does not cause the release of Ca^{2+} by breaking down the membrane potential or dis-
rupting the integrity of the mitochondrial membrane. Similar results have been ob-
served with mitochondria isolated from heart.

With liver mitochondria the release of Ca^{2+} by PGB_x in the presence of acetate
was inhibited by oxalacetate, but the effect was very inconsistent. Using a variety
of measurement techniques, including experiments with metallochromic indicator mur-
exide, the Ca^{2+} electrode and $^{45}Ca^{2+}$, the inhibition can be observed. The release
in the presence of acetate is not effected by ruthenium red. It was considered that
perhaps oxalacetate was causing an oxidation of the reduced pyridine nucleotides by
way of the malic dehydrogenase reaction and that perhaps the oxidation-reduction
state of the mitochondria might be important in the maintenance of the intra-mitoch-
ondrial Ca^{2+}. Addition of various oxidants, including acetoactive and phenazine meth-
osulfate, however, did not have any effect on the Ca^{2+} release with PGB_x, even though

these agents caused a rapid oxidation of pyridine nucleotides. Neither malate nor asparate, both products in the metabolism of oxalacetate, had an effect and direct measurements of Ca^{2+} uptake in the presence of PGB_x into an organic solvent demonstrated that oxalacetate itself did not bind Ca^{2+} and prevent the movement of Ca^{2+}.

With phosphate as the permeant anion, the kinetics of Ca^{2+} released by PGB_x were dramatically different from those observed in the presence of acetate. With acetate, Ca^{2+} is released immediately upon addition of PGB_x and was not effected by ruthenium red. In the presence of phosphate, there was a long lag between the addition of PGB_x and the eventual release of Ca^{2+} and the initial rate was very slow and accelerated with time. The maximum rate was only approximately 25% of that in the absence of phosphate but in the presence of acetate. In addition, the release of Ca^{2+} by PGB_x in the presence of phosphate was totally prevented by ruthenium red.

In addition to the effect of oxalacetate and phosphate, ADP was found to prevent the release of Ca^{2+} by PGB_x. The delayed release of Ca^{2+} by PGB_x in the presence of phosphate, was inhibited by addition of ADP.

These results suggest that the release of PGB_x of Ca^{2+} preloaded in mitochondria in some way involves the transport systems for dicarboxylic acids. It is known that the uptake of oxalacetate, phosphate and ADP all involve specific translocases. The addition of butylmalonte, an inhibitor of the dicarboxylic acid carrier, decreased the lag between the addition of PGB_x and the rapid phase of Ca^{2+} release in the presence of phosphate. A possible role of the phosphate-hydroxyl exchange in PGB_x mediated Ca^{2+} efflux was tested with n-ethylmaleimide, an inhibitor specific for this exchange. This inhibitor caused an efflux of Ca^{2+} with a delay of several minutes. The combination of the inhibitor and PGB_x decreased the lag before Ca^{2+} released.

The observation of inhibition by phosphate, oxalacetate and ADP as well as the stimulation by the various inhibitors of transport system suggest that PGB_x does

not act like a classical ionophore, such as A23187. This was confirmed by doing
comparative studies with PGB_x and A23187.

An evaluation of the effect of PGB_x on Ca^{2+} release with isolated heart mit-
ochondria is under way. Hereto, the results suggest a much more complex activity for
PGB_x than that of a simple Ca^{2+} ionophore. One interesting observation is that with
low concentrations of Ca^{2+} ionophore A23187, the addition of PGB_x at low concent-
rations will cause a stimulation of release but at high concentration will cause
an inhibition of release by A23187. In the presence of acetate, a similar inhibition
can be observed but in this case the PGB_x does serve as an active ionophore for cal-
cium. However, the actual amount of Ca^{2+} released is less in the presence of PGB_x
than that maximally observed with A23187. These results would suggest that the PGB_x
is in some way controlling the availability of intramitochondrial Ca^{2+}. The further
complication is that it has now been observed that PGB_x will bind with ruthenium red,
a compound which reacts with glycoproteins and serves as a specific inhibitor of
Ca^{2+} influx into mitochondria. Preliminary results suggest that ruthenium red will,
under certain conditions, specifically bind PGB_x thus preventing it from acting.
This is not a simple interaction in that under many condition we have been able to
observe that even in the presence of ruthenium red, PGB_x still functions as a Ca^{2+}
ionophore.

All of these results indicate the complexity of this type of compound which may
very well be a model for a naturally occurring control mechanism of intracellular
homeostacis.

(3) Bivalent Cation Interaction with PGB_x

As reported previously, PGB_x is an effective ionophore for Ca^{2+} in both naturally
occurring and synthetic membranes. (Ohnishi, S.T. and Devlin, T.M. op.cit.) The
cation specifically for the ionophoretic activity had been determined and was found
to be $Sr^{2+} > Ba^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. (Figure 3). The activity was approximately two
orders of magnitude less active than the very potent bacterial ionophore A23187.

The ionophoretic property has been evaluated in a study of the binding of divalent cations to PGB_x utilizing the observation that the intrinsic fluorescence of PGB_x is quenched on addition of the divalent cation and by measurement of cation binding using a metallochromic indicator. The water solubility of the sodium salt of PGB_x permits evaluation of the binding in aqueous solutions by conventional physico-chemical methods.

A similar study was conducted at the NADC and results on cation binding are also reported under a later section. The binding of a variety of divalent cations, La^{3+} and H^+ all caused a quenching of the intrinsic fluorescence. Interaction of the monovalent cations, Na^+ and K^+ did not cause quenching. The order of the quenching affinity, as measured by comparing the concentration of divalent cation to cause 50% quenching was $Zn^{+2} > Co^{+2} > Mn^{+2} > Cu^{+2} > Ca^{+2} > Ba^{+2} > Sr^{+2} > Mg^{+2}$. A similar binding affinity was found by the indicator titration method. A similar quenching can be observed in the presence of La^{3+} and by alteration of the pH as shown in Figure 4. Of some interest is the observation that the addition of K^+ caused an inhibition of the quenching of the fluorescence divalent cations. A double reciprical plot of the change in fluorescence versus the Ca^{2+} concentration in the presence and absence of K^+ is presented in Figure 5. The results suggest that K^+ competes with the binding of the divalent cation; studies of the effect of variation in K^+ concentration suggests that 50% inhibition of the quenching of fluorescence with Ca^{2+} occurs at 20mM K^+.

As shown in Figure 4, the half maximum quenching pH was approximately 6.7. This is in good agreement with the pK value determined by the titration method (E. Polis and H. Shmukler, Personal Communication).

Using the metallochromic indicator titration method, it has been possible to determine that the molecular weight of PGB_x per one bound Ca^{2+} is approximately 700. Similar values were found for the other divalent cations but for La^{3+} the molecular

weight of PGB_x per one bound trivalent cation was estimated to be about 1050. This result would suggest that 2 molecules of the monomer (minimal function unit of molecular = 350) are required to bind each divalent cation. Thus, a PGB_x molecule with an approximate molecular weight of 2,200 would be capable of binding 3 Ca^{2+}. The minimal complex unit for Zn binding was apparently different from that of Ca^{2+} binding and measurements of possible cooperativity between binding sites suggests some degree of cooperativity occurring in the binding of Zn^{2+}.

These results are of particular significance in that they demonstrate the possible interaction of Ca^{2+} and K^+ and the role of the pH. It could be that some of the difficulties in achieving constant reproducible results for the effects of PGB_x in biological systems could be due to either competition of monovalent cations for binding sites on PGB_x or possible changes in activity due to microenvironmental changes in pH. It is possible that the slight stimulatory effects of PGB_x on respiration reported in 1980 could be due to a slight uncoupling due to a breakdown of the proton gradient across the mitochondrial membrane. These studies have been prepared for publication and will be submitted in the near future.

(4) Perfused Heart Studies

In 1980, we reported preliminary studies on the effect of PGB_x on the mechanical activity of an isolated perfused rat heart preparation. A recirculating perfused rat heart system has been established in which the rate and amplitude of cardiac mechanical activity is measured. The isolated rat heart was perfused with a modified Krebs-Ringer buffer and oxygen tension was monitored with a Clark electrode. Mechanical activity of the heart was measured through a tension transducer with a strip recorder. As indicated in Figure 6, when the perfusion was stopped the heart became anoxic and there was a stop in the mechanical activity. The assay procedure involves a period of time of approximately 20 minutes for stabilization of the heart activity and at time zero perfusion was stopped. As indicated, shortly after time zero the activity decreased to 0. If perfusion was reinitiated at various times after time

zero, the heart activity was reinstituted if the time was not excessively long. In most experiments at 30 minutes there was no return to normal mechanical activity.

As demonstrated in the bottom of Figure 6, PGB_x when added to the perfusate 7 minutes prior to stopping the perfusion, protected the heart during the anoxic period in that it was able to reinstitute mechanical activity when perfusion was reinitiated. The degree of return to normal amplitude was measured in the individual hearts as a percent recovery. The effects of variation of length of time of anoxia has been measured in both control and treated hearts. In most cases, untreated hearts will not return to normal activity if permitted to be anoxic for 30 minutes or more. Figure 7 presents the results of one series of experiments on the effect of PGB_x on the anoxic heart. The percent recovery of the amplitude of cardiac activity versus the time of anoxia in minutes is presented. At 30 minutes, most hearts did recover if pretreated with PGB_x; with increasing times of anoxia the percent recovery of activity decreased in the treated hearts. The studies indicated, however, that the system was much more difficult to control than anticipated. We observed that there was a good deal of variation in the recovery of both the untreated and treated hearts and that it was very difficult to quantitate these changes. A series of studies have been carried out to evaluate the perfusion system; it has been observed that if the pH was lowered from 7.5 to 7.1, that there was no protection by the PGB_x. We have attempted to utilize other buffer systems besides HEPES and currently are conducting a series of controls using a bicarbonate-CO_2 system.

The evaluation of PGB_x in the system is somewhat tedious because only one heart can be measured at a time, for a maximum of two or three a day. Thus, it is difficult to conduct enough experiments in a short time to eliminate variations due to animal differences, perfusion system, etc. A major effort has been placed on evaluation of the system and it has been necessary to defer careful evaluation of various PGB_x concentrations. It it planned to evaluate several biochemical parameters, includ-

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ing lactic acid production in the anoxic heart, rate of oxygen utilization during development of anoxia and measurement of loss of intracellular components, to evaluate the degree of tissue necrosis that might be occurring. In addition to technical difficulties with the system, we have also had a turnover of technical personnel requiring a period of training of individuals to carry out these experiments. We have also been concerned about the possibility of changes in the PGB_x samples over the past nine months. Even though we have no convincing evidence that there has been a change, there have been periods when we have been unable to demonstrate a positive PGB_x effect in the perfusion system. Measurements of the protective effect of these samples on isolated mitochondira, however, indicated no change in activity.

The results even though incomplete, and not yet statistically evaluated, do indicate a protective effect of PGB_x on the isolated anoxic heart.

(5) Effect of PGB_x On Development of Stress Ulcer in Rats

With the cooperation of Drs. K. Kumashiro and T. Matsumoto, Department of
Surgery, Hahnemann Medical College and Hospital, the effect of PGB_x on the
development of stress ulcers in rats was evaluated. Drs. Kumashiro and Matsumoto
have been utilizing a model system for evaluating the various parimeters in
development of a stress ulcer, (Kamarada, Y., Weiss, R., and Matsumoto, T.,
(1975), Am. J. Surg., 129, 249). There is a very high morbitity and mortality
secondary to stress ulcers in critically ill patients. These investigators have
developed a simple model in which a rat is placed in a restraining cage slightly
larger than its body size and plugged at both ends with a rubber stopper. The
cage is placed in a head down position in a cold room at 4°C for 3 hours and
the animal is sacrificed immediately. After specified surgical treatment, the
stomach is removed and fixed for scoring of gastric bleeding and ulceration.
The severity of gastric mucosal bleeding is catagorized into 4 degrees according
to the quantity of blood clots covering the surface of the glandular part of the
stomach (score 0 - no blood clot; score 1 - several scattered blood clots; score 2 -
more than half of the glandular mucosa covered with blood clots and score 3 -
almost the entire surface of the mucosa covered with clots). The severity of
gastric ulceration is estimated after the removal of blood clots from the gastric
mucosa and scored in a similar fashion. In addition, blood platelet count and
gastric acid secretion are evaluated. As shown in Table I the bleeding score in
the groups given PGB_x in doses of 1 to 5 mg/kg were significantly less than that
of the control groups given saline. The ulceration scores at 1 mg/kg and 5 mg/kg
in the PGB_x groups were 6.21 ± 6.74 and 5.96 ± 5.6 respectively while those of the
control group was 14.42 ± 8.82 (Table II). Statistical analysis of the results
indicate a significant decrease in ulceration and in bleeding scores for the PGB_x
treated rats. The treated animals did not manifest any change in the hematocrit
and platelet count. The gastric acid output of the 1 mg/kg PGB_x group was

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0.236 $\pm$ 0.173 meq/three hours and the control group was 0.189 $\pm$ 0.139 per

three hours. The t test indicated that there was a significant increase in acid

secretion in the PGB_x treated group.

These results demonstrate that PGB_x has a protective effect on ulcer formation

and gastric bleeding in this model. It has been reported (A. Robert, et. al., (1979),

Gastroenterology, 77, 433) that prostaglandins A, E and F have a gastric cytoprotective

effect, indicating that these prostaglandins protect the mucosa of the stomach

and intestines from becoming inflamed and necrotic when the mucosa is exposed

to noxic agents. The secondary effects of the various prostaglandins, however,

preclude their effective pharmacological use in this condition. PGB_x is as

effective as any other single compound tested in the stress ulcer model system.

It is of interest that ulceration has been considered to be due to an increase in

gastric acid secretion and that inhibition of acid secretion would partially

prevent ulceration from occurring. PGB_x stimulated the acid secretion at the same

time as reducing ulceration. The specific causes of stress ulcer formation are

unknown but it is possible that changes in the microcirculation, leading to ischemia

might be involved and thus the protective effect of PGB_x may be due to a prevention

of the tissue damage during ischemia. It is planned to continue these studies

in attempt to evaluate the possible mechanism of action of PGB_x in this animal

model.

Legends

Figure 1   The release of Ca^{2+} from pre-loaded mitochondria by
PGB_x. Mitochondria (1mg/ml) were suspended in 5 ml of 100mMKcl,
20mM MOPS, pH 7.6, 5mM sodium acetate, 10mM succinate and 4μM
rotenone. To this was added 100 μM Ca^{2+}, and at the completion
of uptake 0.6 μM ruthenum red and PGB_x. The efflux of Ca^{2+} was
measured with a Ca^{2+}-electrode. Each point represents the mean of
at least three separate determinations.

Figure 2   Inhibition of PGB_x - induced Ca^{2+} efflux by oxalacetate.
Ca^{2+} fluxes were measured with a Ca^{2+} electrode in a mixture of
100mM KCl, 20mM MOPS, pH 7.6, 5mM sodium acetate, 10mM succinate,
4uM rotenone and 1mg/ml mitochondria. Oxalacetate (1mM) was added
before ruthenium red (0.6μm) or PGB_x (4.2μM). Numbers in parenthesis
indicate rate of Ca^{2+} release in nmol/min/mg protein.

Figure 3   The fluorescence quenching as a function of the divalent
cation concentration. Each of the solutions contained 10 mM
Tris-HCl (pH 7.8) and 31 μg/ml PGB_x. The titration of the divalent
cation, A; $MgCl_2$, B; $SrCl_2$, C, $BaCl_2$, E; $CuSO_4$, F: $MnCl_2$, G; Co
$(NO_3)_2$, H; $ZnSO_4$.

Figure 4   The pH effect to PGB_x fluorescent intensity. Each of
the solutions contain 10 mM MES; 10 mM HEPES, 100 mM KCl and 24
μg/ml PGB_x. pH was adjusted by adding KOH.

Figure 5   Double-reciprocal plot of the Ca^{2+} titration of the
fluorescence. o, in 10 mM Tris-HCl (pH 7.8); and ●, in 100 mM
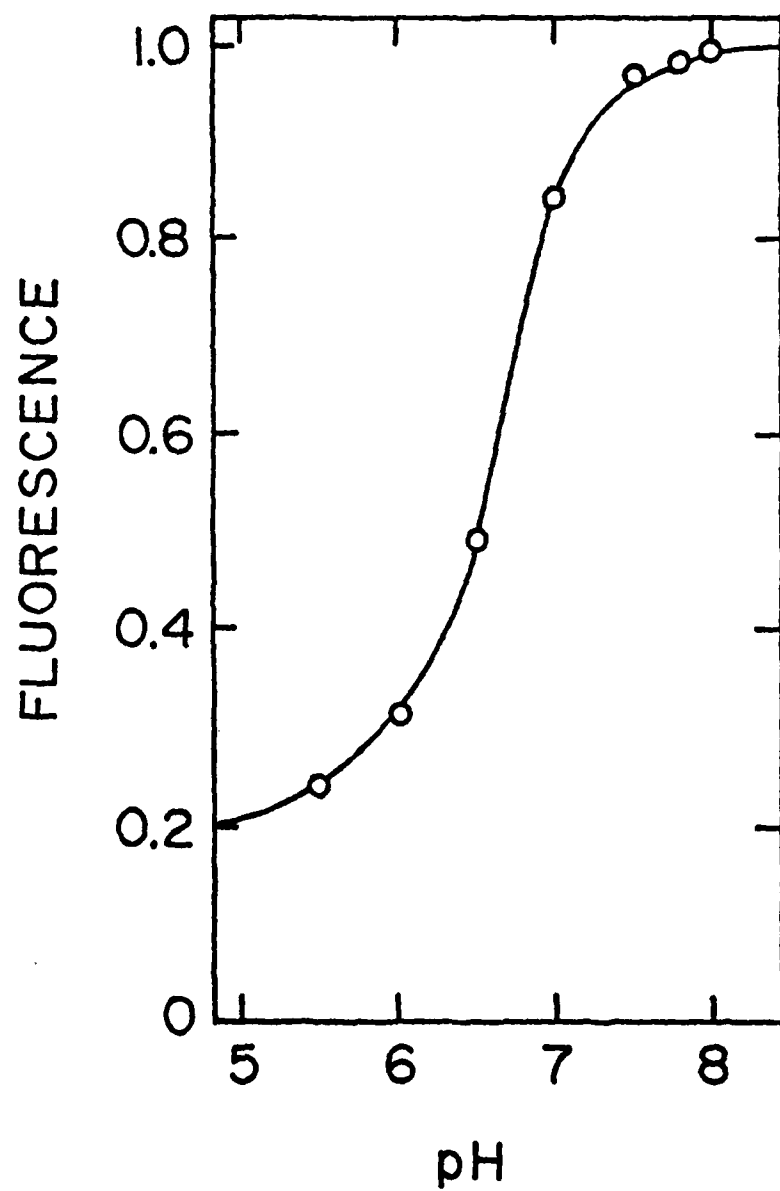KCl and 10 mM Tris-HCl. Each solution contained 31 μg/ml PGB_x.

Figure 6   The amplitude and frequency of mechanical activity re-
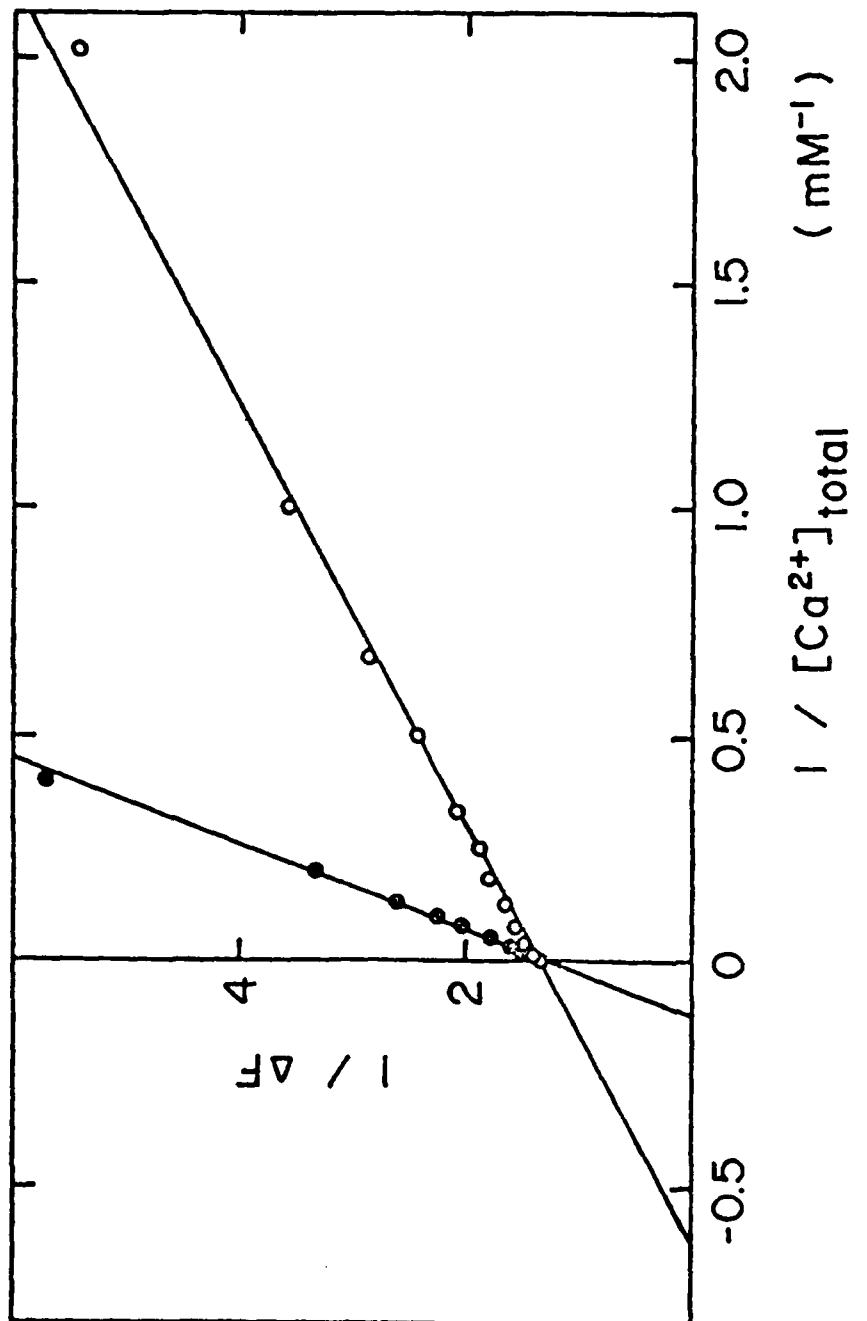corded for a control heart (top) and a heart perfused with PGB_x.

Figure 7   The percent recovery of the amplitude of cardiac
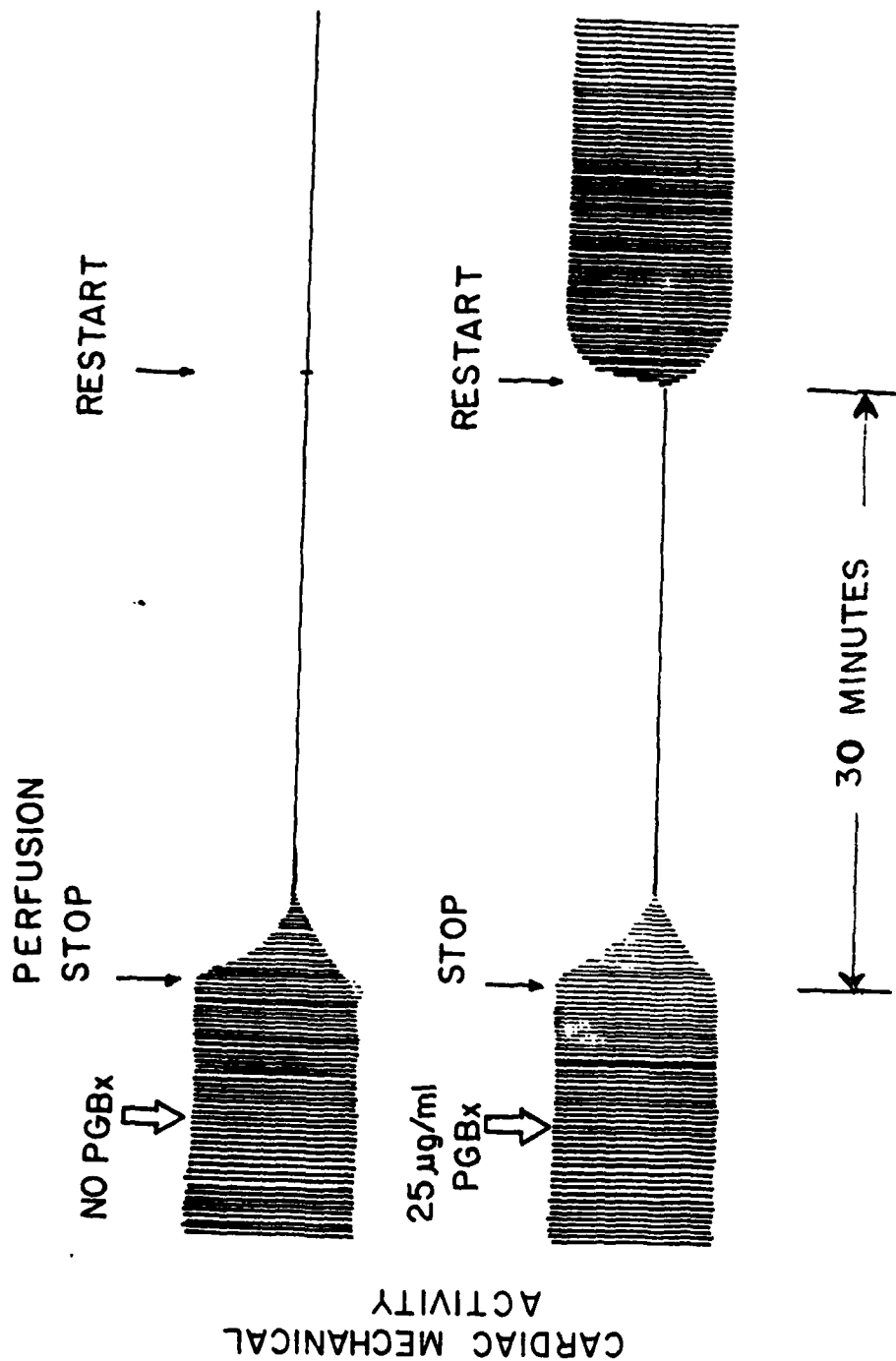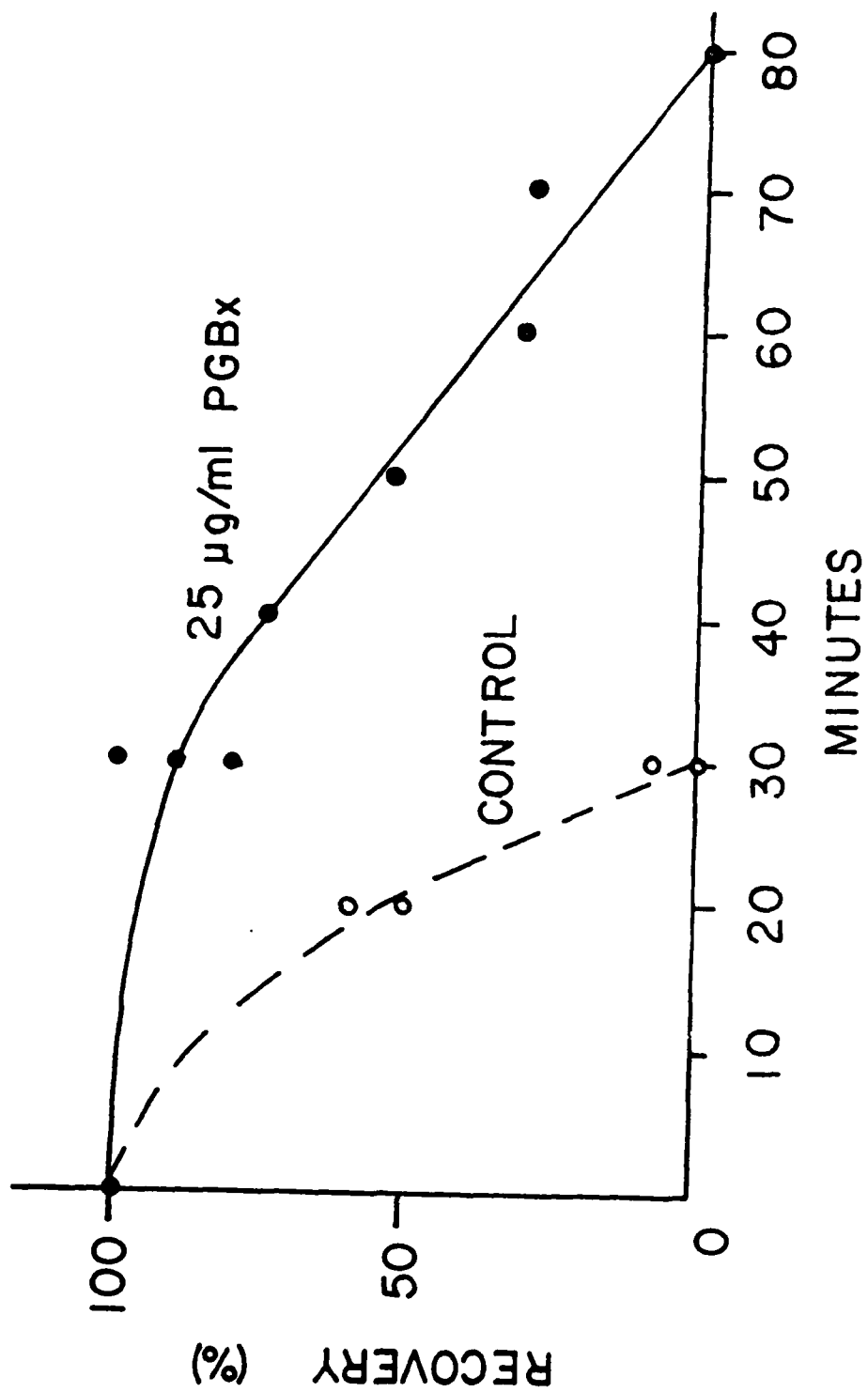activity versus the time of anoxia in minutes.

CARDIAC MECHANICAL ACTIVITY
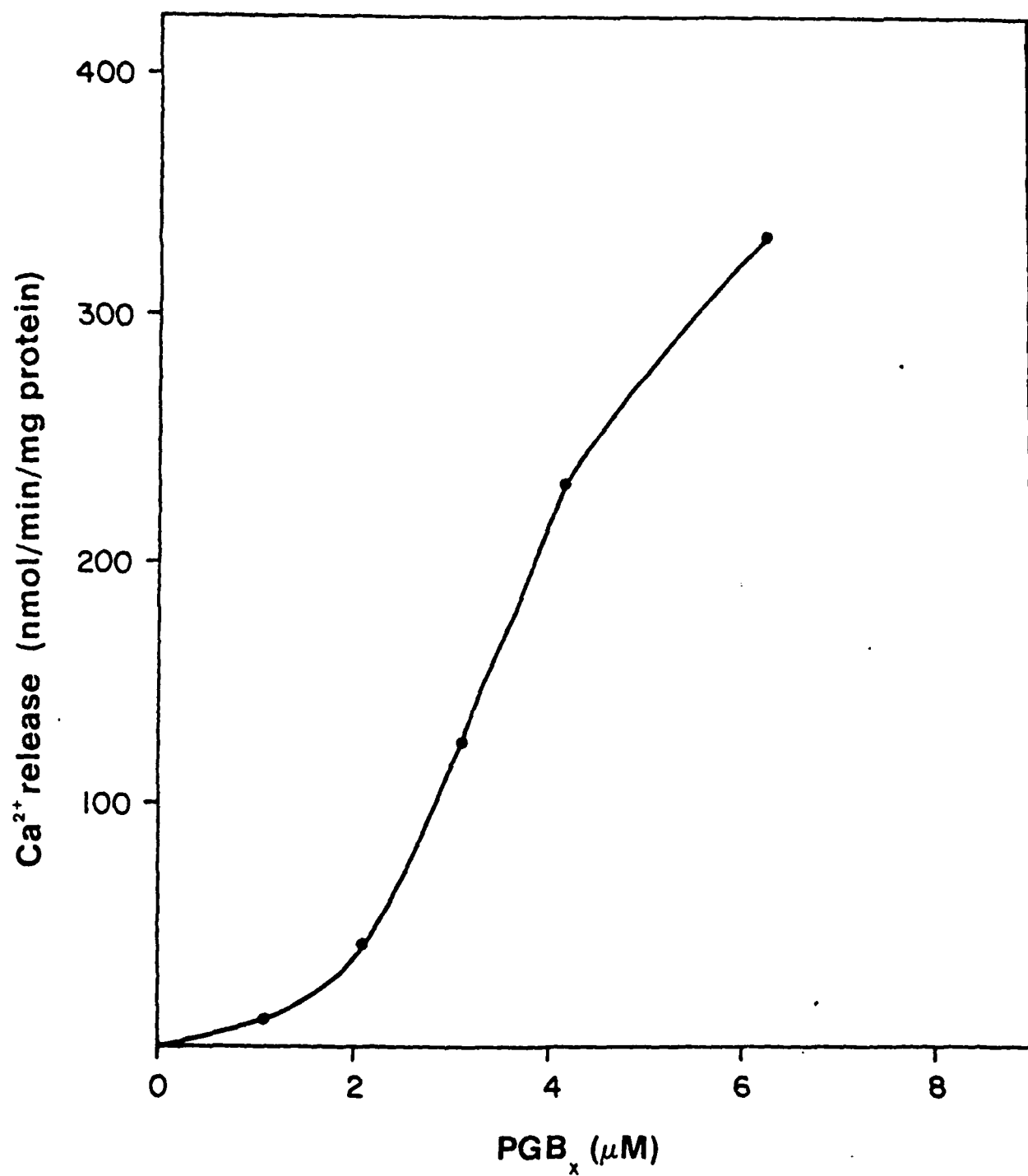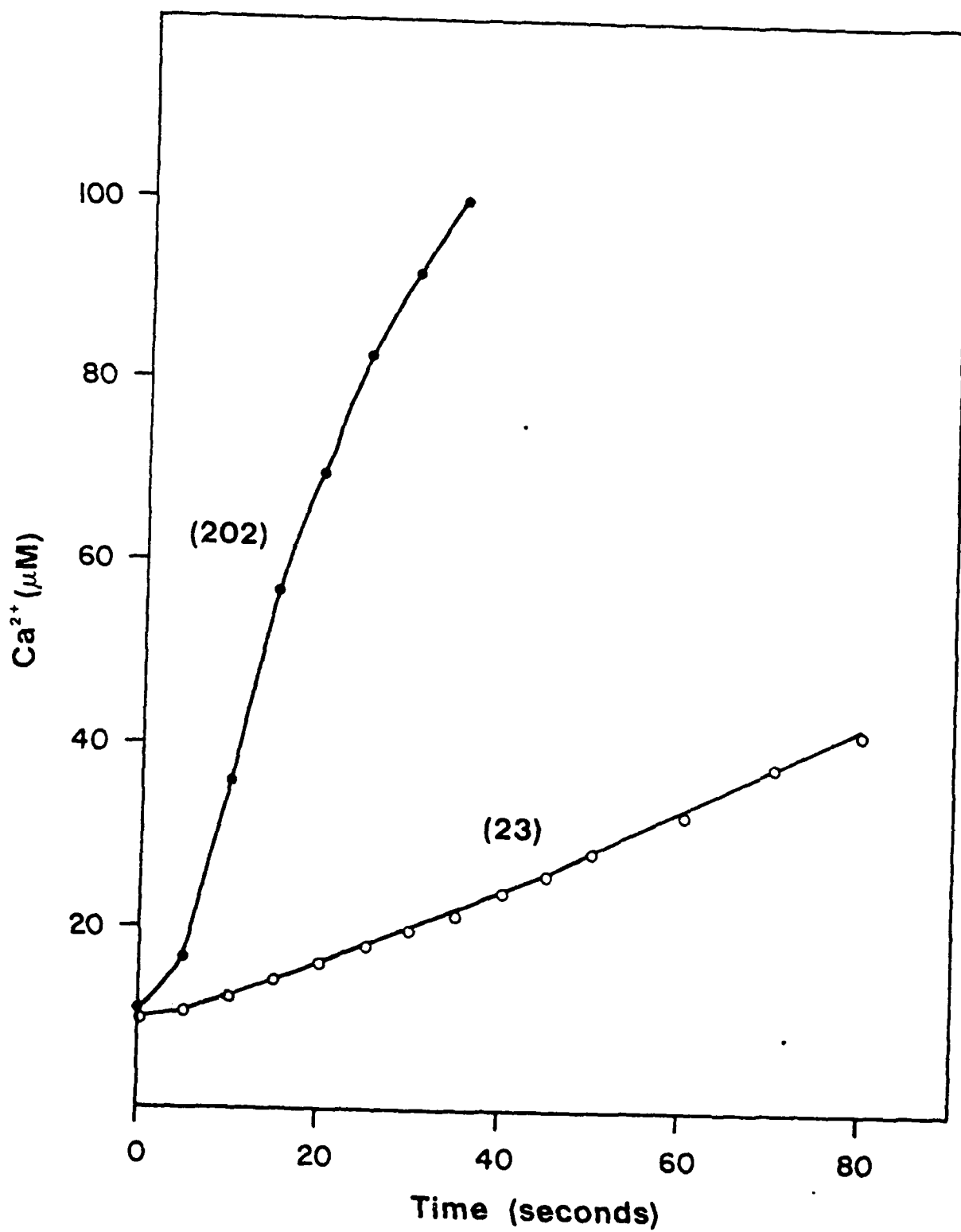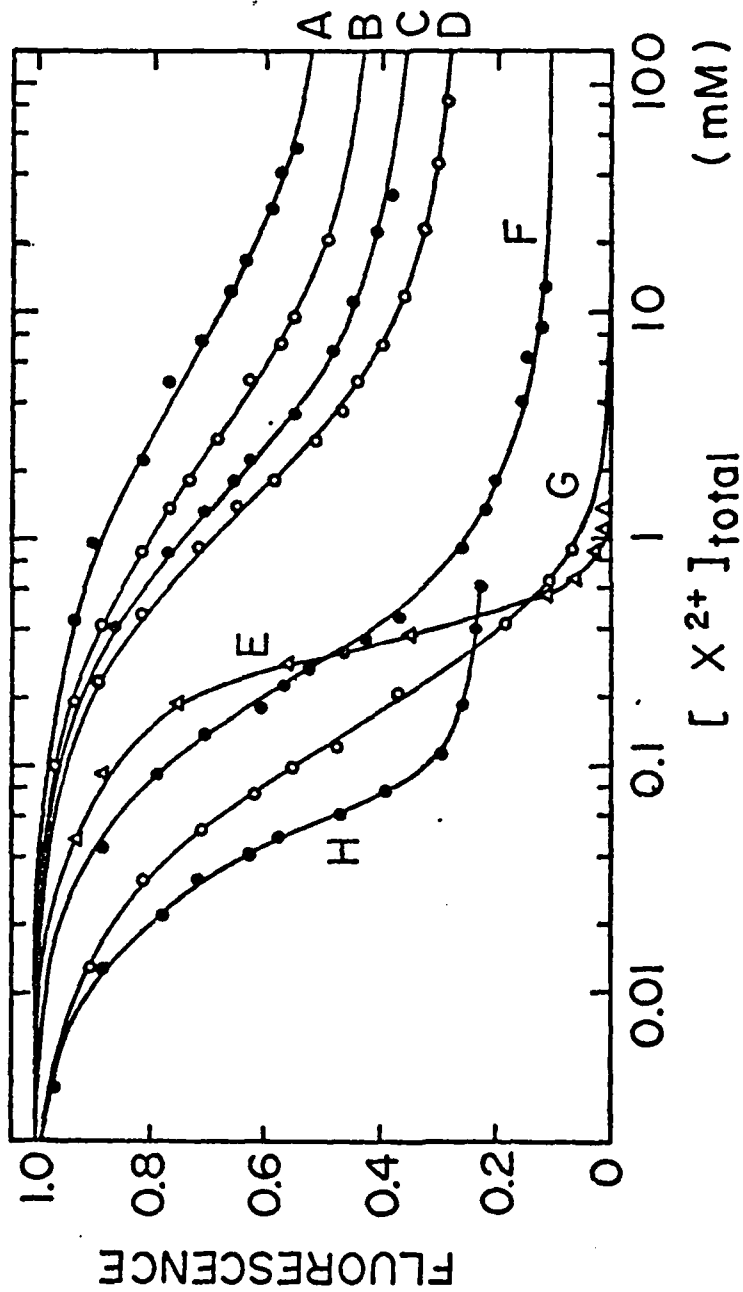
Table 1

THE EFFECT OF PGB_X IN THE DEVELOPMENT OF STRESS ULCERS IN RATS
(BLEEDING SCORE)

BLEEDING SCORE	CONTROL	0.5 MG/KG	1 MG/KG*	5 MG/KG*	25 MG/KG
0	1/40 (2.5%)	0/8	6/41 (14.6%)	1/22 (4.5%)	0/4
1	11/40 (27.5%)	3/8	16/41 (39%)	12/22 (54.5%)	3/4
2	11/40 (27.5%)	/8	16/41 (39%)	7/22 (32%)	0/4
3	17/40 (42.5%)	4/8	3/41 (7.3%)	2/22 (9%)	1/4
TOTAL NO. RATS	40	8	41	22	4

*SIGNIFICANCE (P<0.01 χ^2)

Table 2

THE EFFECT OF PGB$_X$ IN THE DEVELOPMENT OF STRESS ULCERS IN RATS
(ULCERATION SCORE)

EXPERIMENT GROUP		NUMBER	ULCERATION SCORE
CONTROL	SALINE	40	14.42 ± 9.82
PGB$_X$	0.5 MG/KG	8	21.23 ± 20.23
	1 MG/KG	41	6.21 ± 5.74*
	5 MG/KG	22	5.96 ± 5.60*
	25 MG/KG	4	3.75 ± 6.14

*SIGNIFICANCE (P < 0.01 T-TEST)

PART A   SYNTHESIS, PURIFICATION AND MITOCHONDRIAL STUDIES.

I. Purification and stockpiling of 15-keto PGB₁

In collaboration with St. Josephs College, Phila., Pa, in which they car-
fied out the synthetic steps while NADC purified the intermediates, 265 g of
15-keto PGB₁ was stockpiled during fiscal 1981.

II. Synthesis, Purification of PGBₓ

PGBₓ preparations prepared during the past year and remains of preparations
from previous years were pooled in similar type fractions as defined by the seph-
adex LH-20 gel filtration. The stocks of these fractions are as follows:

$$
\begin{array}{lll}
\text{Type I} & : & 23.3\ \text{g} \\
\text{Type II} & : & 32.4\ \text{g} \\
\text{Type III} & : & 16.7\ \text{g} \\
\text{Type IV} & : & 33.3\ \text{g} \\
\text{Type V} & : & 42.5\ \text{g} \\
\text{Type VI} & : & 30.2\ \text{g} \\
\text{Type VII} & : & 6.7\ \text{g}
\end{array}
$$

All these preparations are available for testing, however, we have been ship-
ping Type II only to fill requests.

III. Further fractionation of PGBₓ into less heterogeneous fractions

It is well established that PGBₓ prepared according to Polis et al is a
heterogeneous mixture of oligomers of varying molecular weights and configuration.
In order to delineate the chemical structure of PGBₓ it is absolutely necessary
to obtain a homogeneous preparation. At NADC we have undertaken a number of
purification steps utilizing various types of chromatography to separate PGBₓ in-
to various fractions. Some of the more successful methods are:

1. Gel filtration on sephadex LH-20 with methanol as the carrier (see fig-
ure 1) this is the method developed by Polis et al. The arrows indicate the
fractions that were separated and are indentified by the numbers indicated.
Fraction 2 is the PGBₓ-Type II that has the best in vitro activity and is the

fraction dispensed to the various ONR contractors for testing. This fraction exhibits the biphasic in vitro PGB_x effect as shown in figure 2.

2. Isolation of a fraction from Type II PGB_x that has a trace amount of PGB_x inhibitor factor: When PGB_x is dialyzed against phosphate buffer at pH 6.5 about 10% of the material dialyzes through the bag. This material when assayed in the in vitro system gives an activity concentration response curve as shown in figure 3, by the dashed line. The importance of this separation is that we now have a preparation of PGB_x equivalent in activity to Type II PGB_x yet most of the inhibitor properties are removed. (Published as Report No. NADC-79183-60)

3. Fractionation of PGB_x by Gel filtration:

Gel filtration in methanol does not yield clean fractions. However, when PGB_x is chromatographed on sephadex G-100 or LKB AC-54 in aqueous phosphate buffer at pH 7.0, two clean fractions were obtained as shown in figure 4. (The two curves shown here are the curves obtained by monitoring the chromatographic effluent at 2 different wavelengths, i.e. 254 nm and 280 nm.) The molecular weights (by vapor pressure osmometry) were about 2400 for the first fraction and 1540 for the 2nd fraction. By combining the techniques of dialysis and gel filtration, PGB_x could be separated into five fractions that differed markedly in their molecular weights and PGB_x activity. Figure 5 shows the schematic representation for the separation of the 5 fractions and the molecular weight of the starting material and the separated fractions. Note that the starting material was PGB_x-Type III that has a lower molecular weight than Type II. The PGB_x activity was distributed in all fractions, however fraction 3 showed a 4 fold increase in PGB_x activator with only a slight reduction in the inhibitor activity, while fraction 1 showed a reduced activator content but a five fold increase in the inhibitor factor. (Publications for these studies are NADC Report No. 79008-60, No. 79085-60, No. 79182-60, No. 80156-60 and No. 80183-60.).

4. Separation of PGB_x by electrofocusing:

Electrofocusing is a technique that separates charged particles according

to their isoelectric point. This is accomplished by carrying out electrophoresis in a pH gradient so that when the amphoteric substance migrates to the point equivalent / to its I.E.P., it stops. Obviously this technique is not applicable to PGB_x since it is anionic in character only. However, separations are obtained in a buffer gradient between pH 3.5 - pH 9.0 by virtue of the PGB_x migration to an acid pH at which point it is insoluble and is no longer charged. Alternatively one can carry out the electrophoresis in a pH gradient in which PGB_x is soluble, such as between pH 7-9. Figure 6 shows just such a separation. My interpretation of this separation is that gel filtration is taking place since the support is Sephadex G-75 and the "carrier buffer" is the electromotive force applied. We obtained 3 fractions which when assayed in the in vitro system gave PGB_x concentration curves as shown in Figure 7. Band 1 the slowest moving fraction had practically no activity while Band 2 exhibited a PGB_x activation almost equivalent to that of the starting material. Band 3 on the other hand showed a marked increase in PGB_x activity that almost reached the values of our standard PGB_x preparation. The results were similar to those obtained with the previous molecular sizing methods.

5. Separation by Chromatography on Hydroxylapatite

Hydroxylapatite is a form of calcium phosphate first described by Tiselius for use in protein separations. We reasoned that since PGB_x is a polymer that it too might be separated on hydroxylapatite using a very dilute phosphate buffer gradient. We were able to fractionate PGB_x by chromatography on columns of Hydroxylapatite equilibrated with water and then shifting to a step-gradient of phosphate buffer from 5×10^{-3} M to 5×10^{-2} M and finally to 0.1 M. Figure 8 curve B shows the chromatogram of a Type III PGB_x. That the separated peaks are real, were proven by carrying out preparative chromatography and rechromatographing the separate fractions. Although the fractionation was not clean, the fact that enrichment of the various fractions when analyzed showed that this

indeed is a chromatographic separation rather than solubility effect often found when PGB_x is chromatographed in non-polar solvents such as chloroform. The lower curve of Figure 8 is the "buffer only" chromatogram. When the PGB_x activity was measured a marked improvement was found in the fractions that eluted later, while the molecular weight distribution proceeded from low in the first fraction and progressively higher as the chromatography proceeded. (Publication: Physiol. Chem. and Physics in press).

6. Reverse phase separation of PGB_x

Previous reverse phase chromatography on Waters C18 column utilizing acid-methanol as described independently by Polis et al and Bieman gave no separations at all. Recently we have tried this technique using 90% MeOH as a carrier and we have found analytically, that the chromatography resolves at least 7 fractions. (Figure 9). At present we are scaling this method up in order to collect sufficient quantities for further analysis. Preliminary studies show that it is indeed reverse phase chromatography since the fractions are enriched with various components.

IV. Interaction Of PGB_x with cations, serum albumin and rat liver mitochondria (RLM)

One of the major problems that must be solved in the PGB_x program is the mechanism of action of PGB_x in its in vivo effect on hypoxic-anoxic episodes in animals. Obviously at this stage it is premature to undertake in vivo studies along this line in view of the fact that a definitive animal model assay is yet to be established. On the other hand the in vitro PGB_x assay that Polis devised has been confirmed in other laboratories. We have attempted to study the mechanism of PGB_x action at the in vitro level. The report by Devlin that PGB_x has "Calcium Ionophoric" properties has prompted us to investigate whether this property is involved in the in vitro PGB_x effect. It is well known that A23187, a potent Calcium Ionophore does not affect oxidative phosphorylation in fresh or

aged RLM. We therefore have attempted to show that PGB_x interacts with many cations and perhaps one of these may be involved in the PGB_x effect. It is also well known that PGB_x exhibits fluorescence when activated in the UV. We have found that this fluorescence may be quenched by the addition of cations. By measuring the fluorescence intensity of PGB_x solutions in the presence of various cations we have been able to estimate a binding constant for the PGB_x cation in question. Fig.10 & 11 show plots of the fluorescence intensity of PGB_x as a function of the concentration of various cations. Assuming that fluorescence quenching is an indication of complex formation, then the binding constant for the PGB_x-Ca^{++} is rather weak in comparison to the heavy metals such as Fe^{++}, Zn^{++} (Cu^{++} not shown here). If we consider the mid point of the Quench curves as representing the amount of cation required to quench 50% of the PGB_x fluorescence then we find that compared to Fe^{++} or Zn^{++} almost 2 orders of magnitude of Ca^{++} are required. Interestingly enough the strongest quencher studied was ruthenium red (R.R.) a known inhibitor of Ca^{++} efflux from mitochondria. If the function of PGB_x depends on its ability to complex cations then in the mitochondria one would expect only certain cations would be involved, namely, Mg^{++}, Ca^{++}, Fe^{++} or Cu^{++}. In preliminary studies it was found that both Cu^{++} and Fe^{++} are released into the in vitro test medium during the degradation stage of the rat liver mitochondria. When PGB_x was added, these levels of both Cu^{++} and Fe^{++} were reduced, suggesting that PGB_x either binds these cations released during degradation of RLM, or binds to the RLM and maintains membrane integrity preventing the release of these cations.

From our chromatographic studies we have concluded that PGB_x is a very reactive molecule and that in some cases once it is applied to a column it cannot be removed without drastic treatment, e.g. anion exchange chromatography. Since it is well known that fatty acids bind to serum albumin very strongly, and since Polis used bovine serum albumin in the in vitro test system it was of interest to find out if there was an interaction of PGB_x with serum albumin. Two pieces of conclusive experimental evidence proved this. The first, from fluorescence quenching, in which the fluorescence of bovine serum albumin (BSA) (measured at

wavelengths that differed from that of PGB_x fluorescence) was quenched by the addition of PGB_x (figures 12 and 13) which show the fluorescence spectra of BSA and BSA–PGB_x complex (Fig 12) and the quenching of BSA as a function of PGB_x concentration (Figure 13). The other piece of evidence is the effect of BSA on the solubility of PGB_x at pH 5.2 (Figure 14). PGB_x is relatively insoluble at pH 5.2 (–:–· curve). When BSA is added, PGB_x is solubilized until the BSA is saturated and then the additional increments of PGB_x causes a precipitation of the complex. Confirmation of the PGB_x–BSA complex formation was the finding that when PGB_x and BSA are added together before the mitochondria in the in vitro test system, the complex no longer exhibits the characteristic PGB_x effect. The importance of this finding is that it permits an explanation for the manner in which PGB_x, when injected into animals, is carried from the injection site to the target site. Presumably PGB_x reacts with serum albumin and is then transported, similarly as other fatty acids, and released by specific enzyme action at the site. As yet this interpretation is only a speculation.

This binding of PGB_x to BSA suggested that perhaps PGB_x also binds to RLM. Since at present there is no method for the measurement of PGB_x at the µg level in the presence of other UV absorbing material, ^{3}H–labelled PGB_x was synthesized in order to increase the sensitivity of detection. Unfortunately [3]H–15–keto–PGB_1 is not available commercially, but since ^{3}H–PGB_1 is, we converted this prostaglandin to its alkaline polymer using a precedure worked out specifically for PGB_1. We then measured the binding of ^{3}H–PGB_{1-x} to RLM during the in vitro PGB_x assay conditions. Figure 15 shows two curves, one labelled P_i esterified, which is the PGB_{1-x} vs concentration curve that showed that the ^{3}H–PGB_{1-x} was as active as our standard PGB_x preparation, and the two line curves, one labelled no nucleotide and one representing the standard in vitro test, which shows the amount of ^{3}H–PGB_{1-x} bound to RLM as a function of added ^{3}H–PGB_{1-x}. As seen here ^{3}H–PGB_{1-x} binds to RLM and this binding increases with increasing amounts of added PGB_{1-x}, and at the higher concentration of PGB_{1-x}, where phosphorylation is inhibited, the binding of PGB_{1-x} to RLM is still increasing. In addition the amount of PGB_{1-x} bound to RLM amounts only to about 20% of the amount added. Although it cannot be concluded that PGB_x derived from 15–keto PGB_1 will behave similarly, the

method used here appears valid and depends only on obtaining a labelled PGB$_x$ preparation.

V. New preparation of PGB$_x$ without inhibitor factor

During the course of oxidizing trans PGB$_1$ to 15-keto PGB$_1$ it was observed that some of the material polymerized. Since this material is in the form of the methyl ester, it was hydrolyzed and then fractionated on the sephadex LH-20 column as carried out routinely in the normal PGB$_x$ preparation. Seven fractions were obtained and assayed individually for PGB$_x$ activity. Figures 16 and 17 are a plot of the PGB$_x$ effect as a function of PGB$_x$ concentration. The results show that standard PGB$_x$ still exhibits the biphasic reaction while the new PGB$_x$, PGB$_{x-ox}$ to distinguish it from the standard PGB$_x$, shows the activation effect equivalent to the standard, while having no inhibitor effect (curves 2,3 and 4 figure 16). Fraction 1 (curve 1) also shows high activation with only a small amount of inhibition. The later LH-20 fractions, fractions 5, 6, 7, and 8 show less activation than standard PGB$_x$ while still having no inhibitor. In order to compare this data with whose of Devlin's group the concentration of PGB$_x$ used must be corrected by a factor of 3. In other words, the fraction tested at 80 µg PGB$_x$ is equivalent to 240 µg in Devlin's system. Fractions 2 and 3 were also tested with fresh non-degraded RLM and no inhibition over the concentration used was observed. Preliminary measurements indicate that the molecular weight of fraction 2 is much less than that of PGB$_x$-Type II probably about 1500 daltons. Obviously this implies that PGB$_{x-ox}$ is a less complex oligomer than standard PGB$_x$ and exhibits only one type of effect on RLM--activation. We expect to stockpile this material and should have 1-2 grams available for further testing.

PGB$_x$ papers published by Biochemistry Research Team under Dr. H. W. Shmukler's

Supervision

1. B. David Polis, Sara F. Kwong, Edith Polis, George Nelson, H. W. Shmukler. Studies on PGB$_x$ A Polymeric Derivative of Prostaglandin B$_1$: I - Synthesis and Purification of PGB$_x$. Physiol. Chem. and Physics, Vol. 11, 1979.

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4. H. W. Shmukler, E. Soffer, S. F. Kwong, M. G. Zawryt, W. Feely and E. Polis. Studies on PGB$_x$ Isolation of a PGB$_x$ with Reduced Inhibitor Content. Report No. NADC-79183-60, 1979.

5. H. W. Shmukler, S. F. Kwong, E. Soffer and M. Zawryt. The Fractionation of PGB$_x$ by Molecular Exclusion Chromatography. Report No. NADC-79008-60, 21 Mar 1979.

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7. H. W. Shmukler, B. D. Polis and E. Soffer. Urinary Molecular Determinants for the Prediction of Ischemic Anoxic Stress Pathology: Evaluation of Lipid Peroxides, Phemolic Acids and Generated Free Radical Compounds as Bioindicators of Stress. Report No. NADC-79084-60, 15 May 1979.

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9. B. D. Polis, E. Polis. Novel Prostaglandin Derivatives, Certain in Vivo and in vivo effects thereof and Processes for the Preparation of same. United States Patent (19) (11) 4, 153, 808, 8 May 1979.

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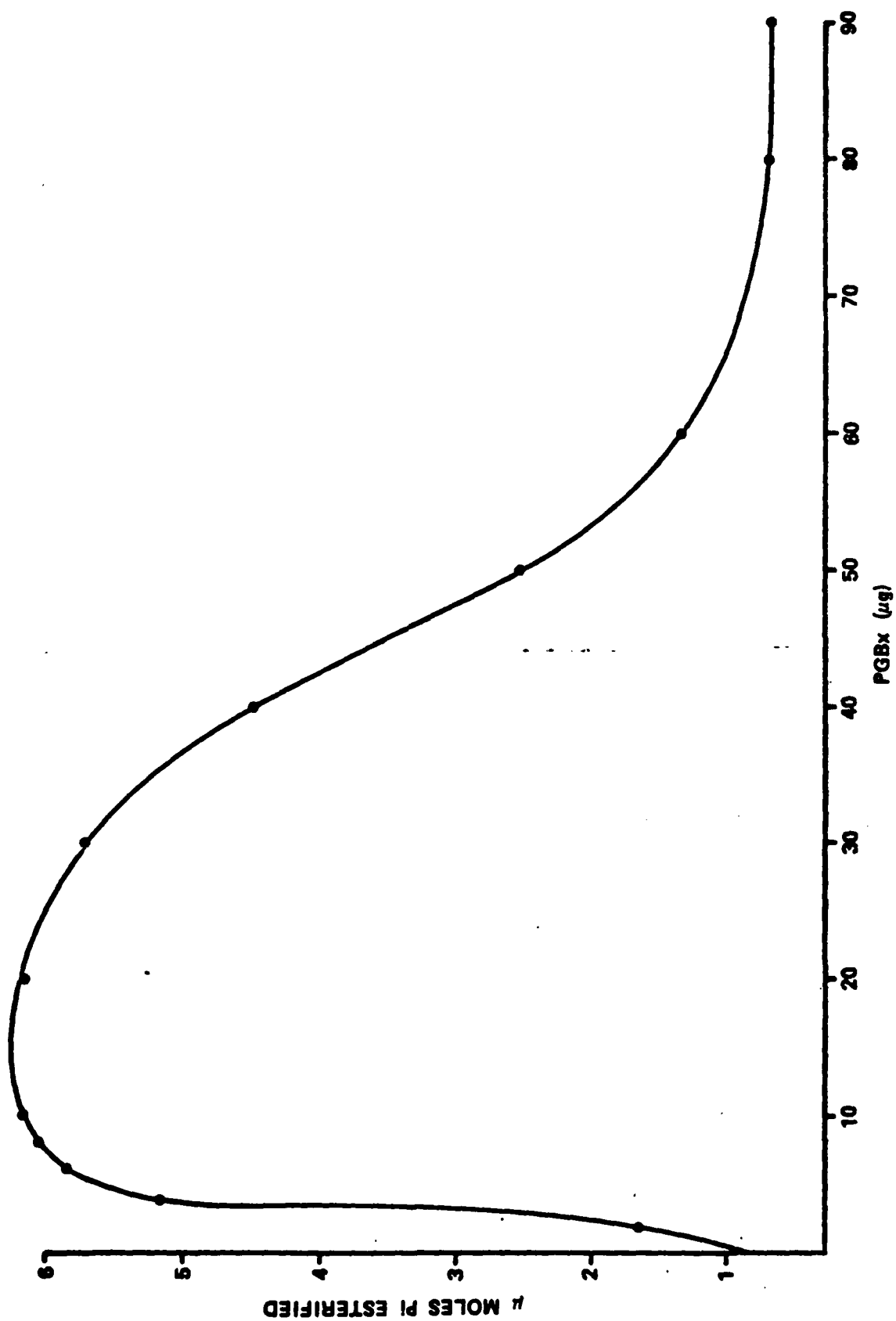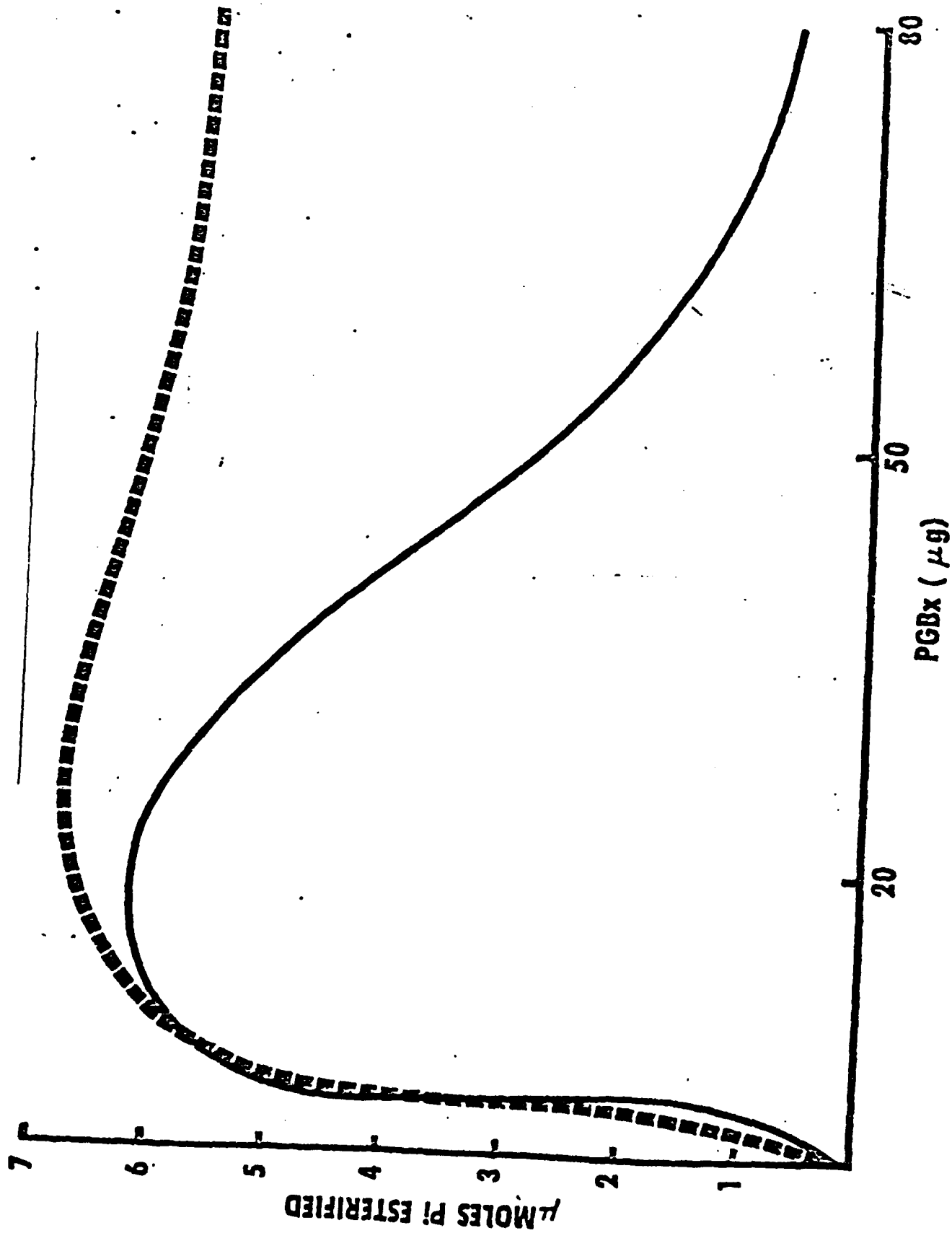
FIGURE 1

FIGURE 2

PGBx (μg)

μ MOLES Pi ESTERIFIED

FIGURE 3

μMOLES Pi ESTERIFIED

PGBx (μg)
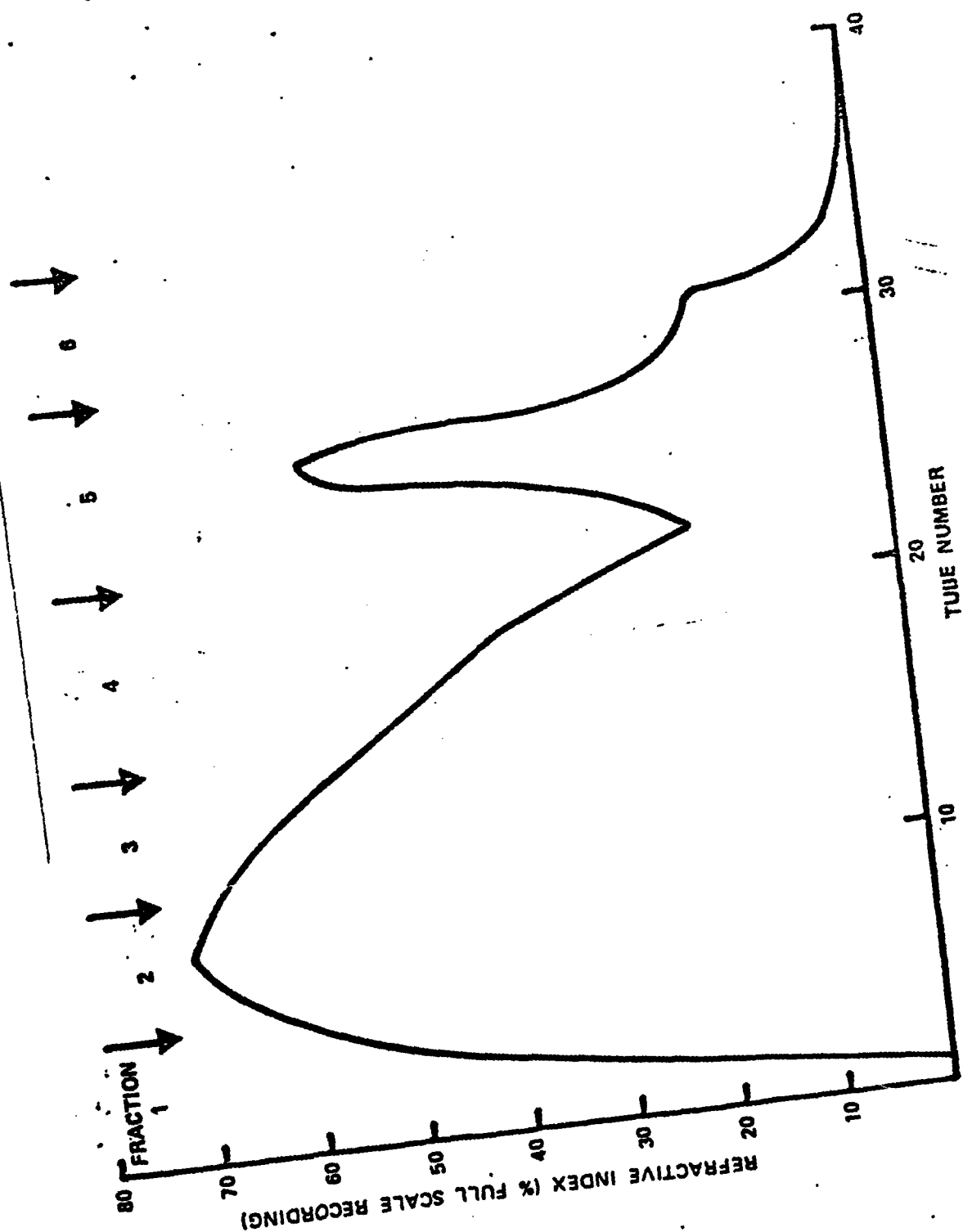
FIGURE 4

PGBx EXCLUSION CHROMATOGRAPHY ON SEPHADEX G—100

A254 NM

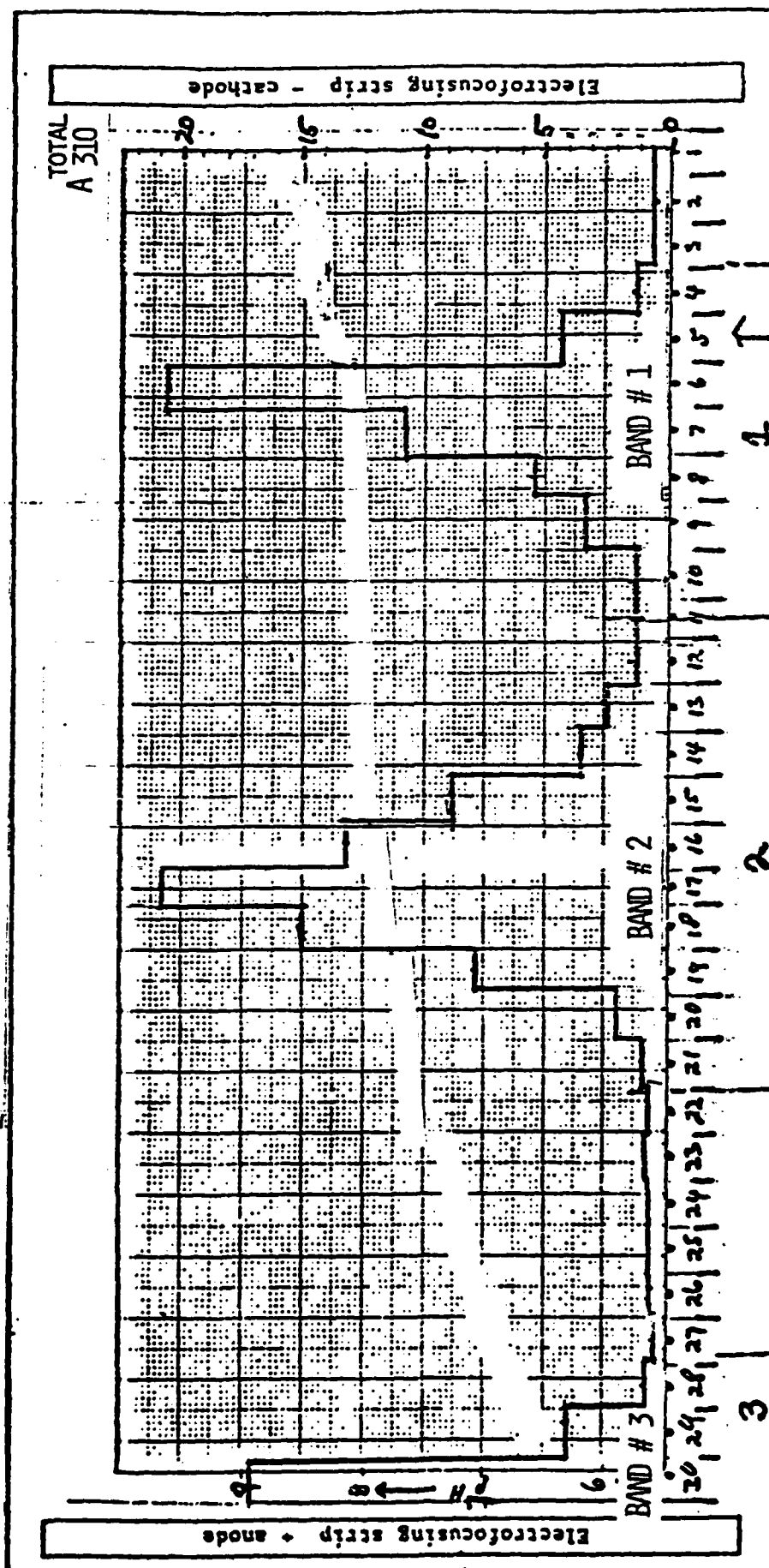A280 NM

ABSORBANCE UNITS

- 45 -

FIGURE 5

PGB$_x$ (FRACTION III)

DIALYSED VS PHOSPHATE BUFFER – 12000 d

MW=1749

RETENTATE

DIALYSED VS METHANOL

RETENTATE

FRACTION 5

MW,2296

DIALYSATE

M.E.C.

LOW RETENTION VOLUME

FRACTION 4

MW,2061

HIGH RETENTION VOLUME

FRACTION 3

MW,1789

DIALYSATE

DIALYSED VS PHOSPHATE BUFFER–8000 d

RETENTATE

FRACTION 2

MW,1700

DIALYSATE

FRACTION 1

MW,718
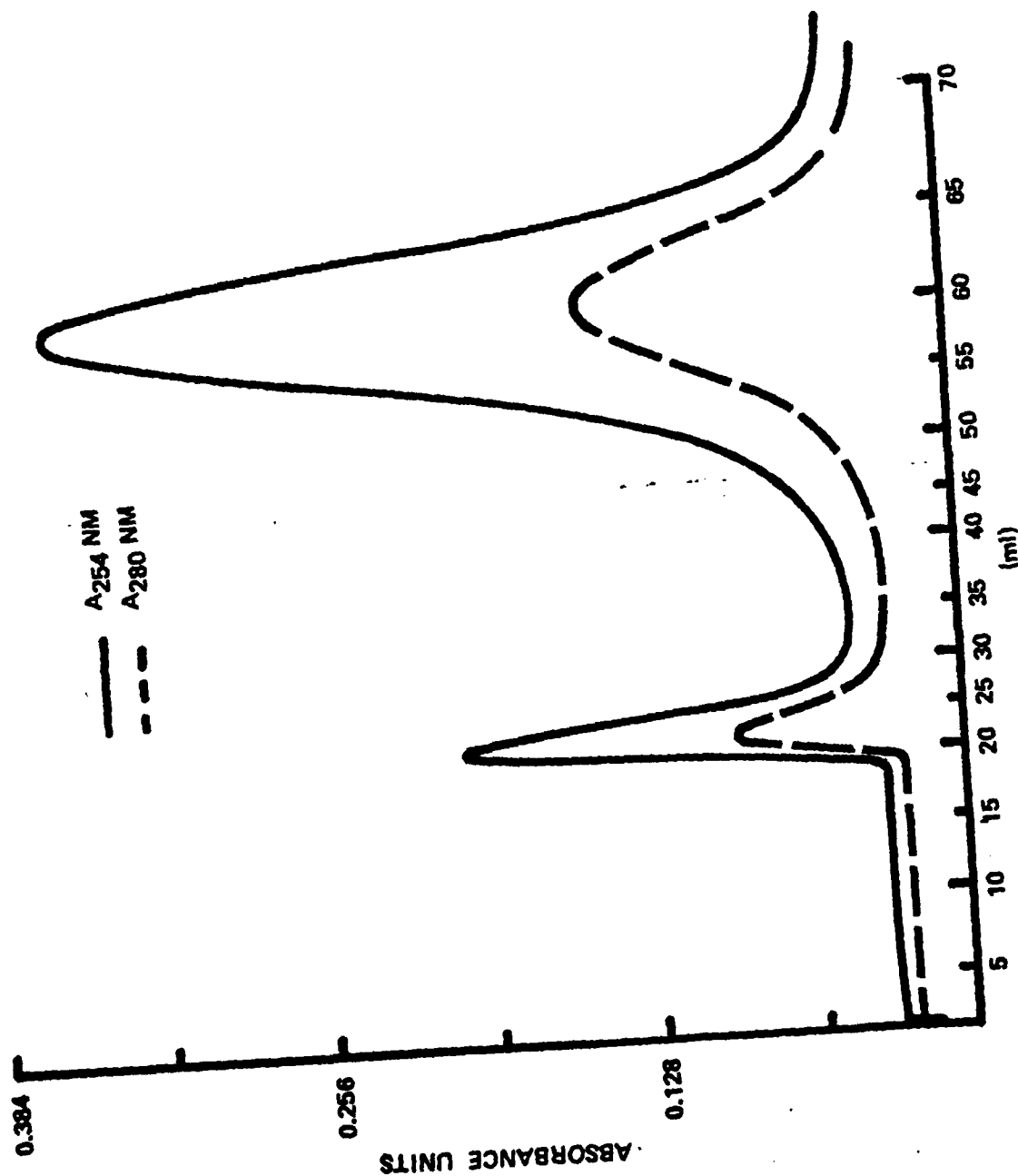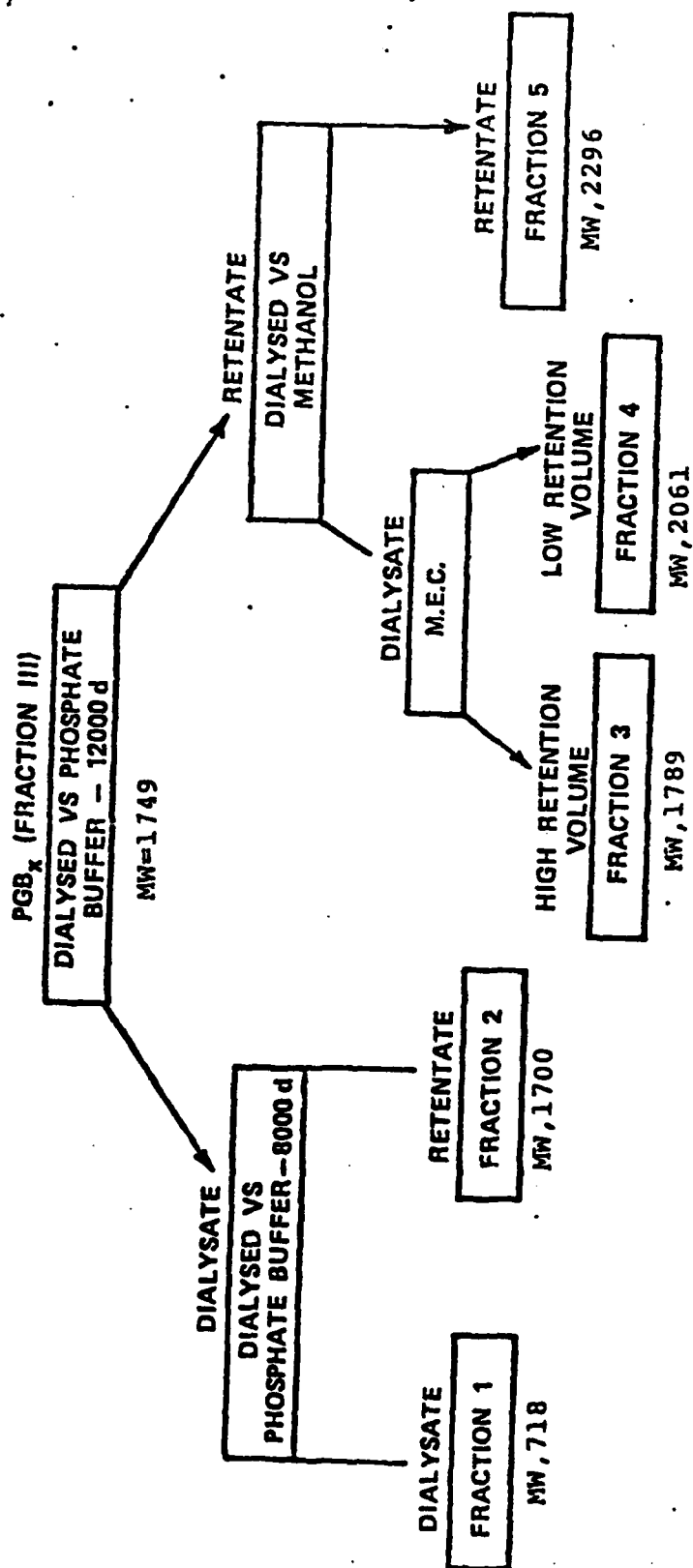
FIGURE 6

ELECTROFOCUSING EXPERIMENT # 13

Fraction: PGBx fraction 3

AMPHOLINE BUFFER pH 7-9

ACTIVITIES OF PGBx FRACTIONS FROM   ELECTROFOCUSING EXPERIMENT.

FIGURE 7
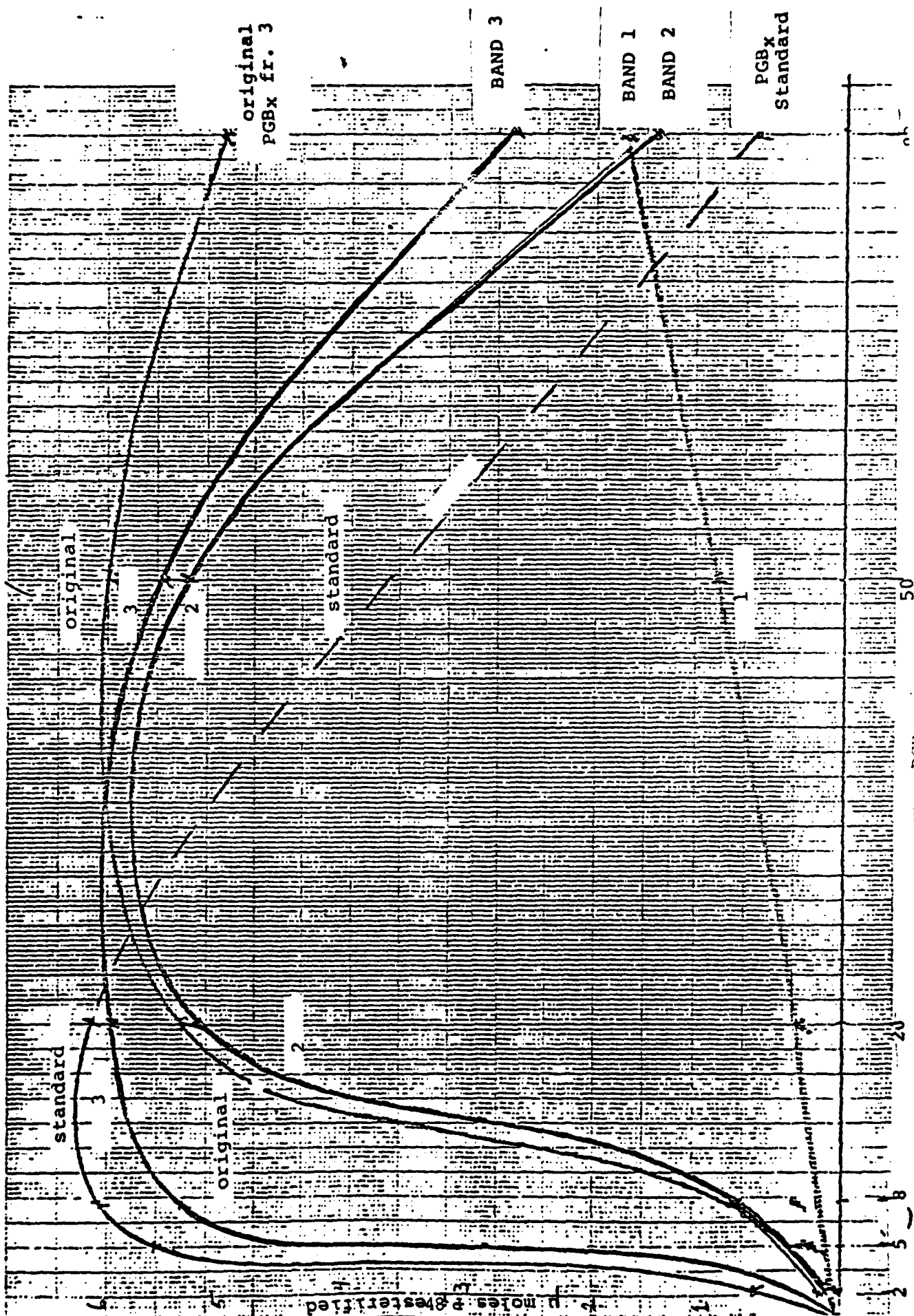
FIGURE 8
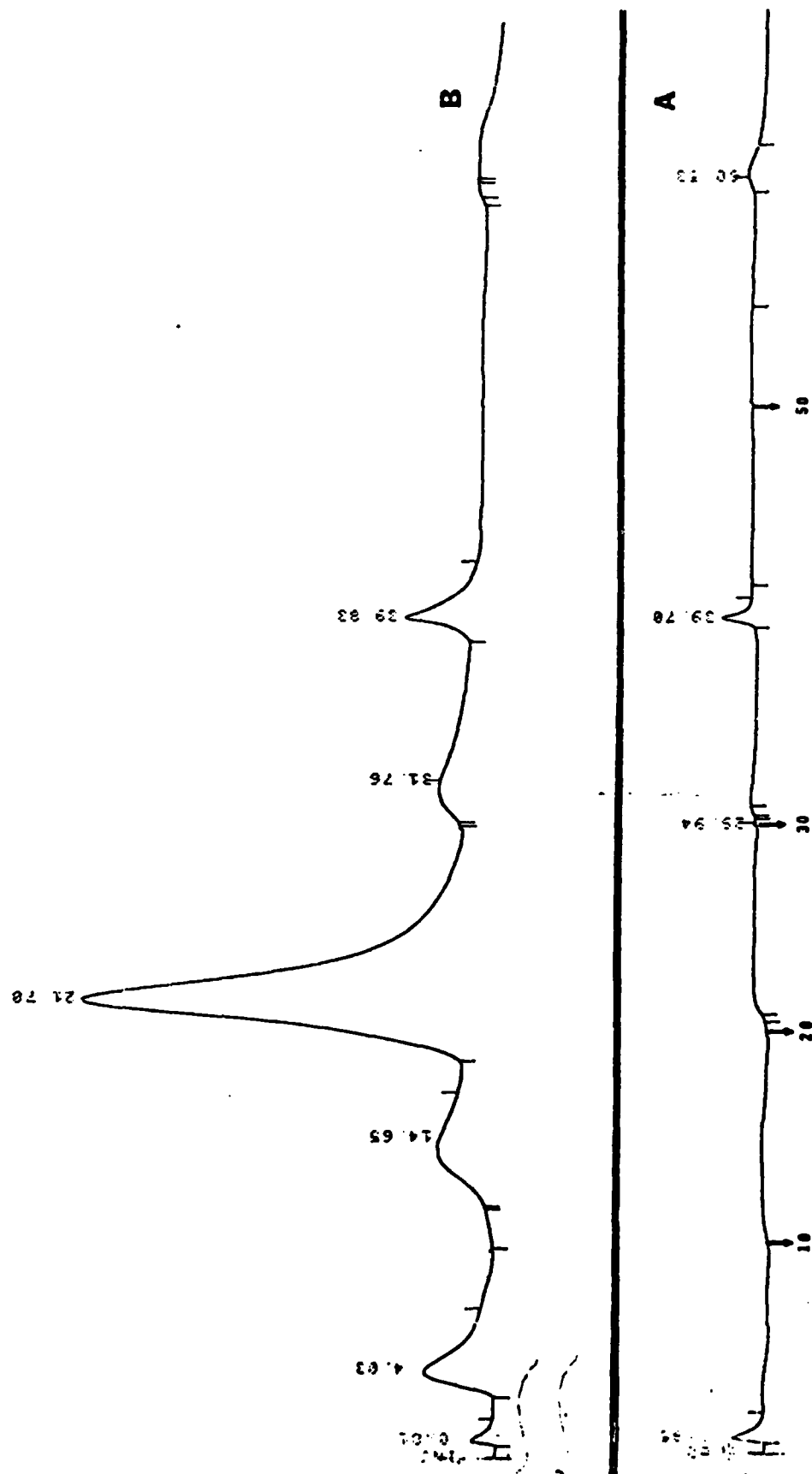
FIGURE 9

DATE 4/6/81 OPERATOR W. Feely CHROM. NO.
COLUMN Waters C18
SOLVENT A H2O COMP
SOLVENT B MeOH COMP
DETECTOR 254 nm SENS 6.4×10⁻⁴ AUFS/cm
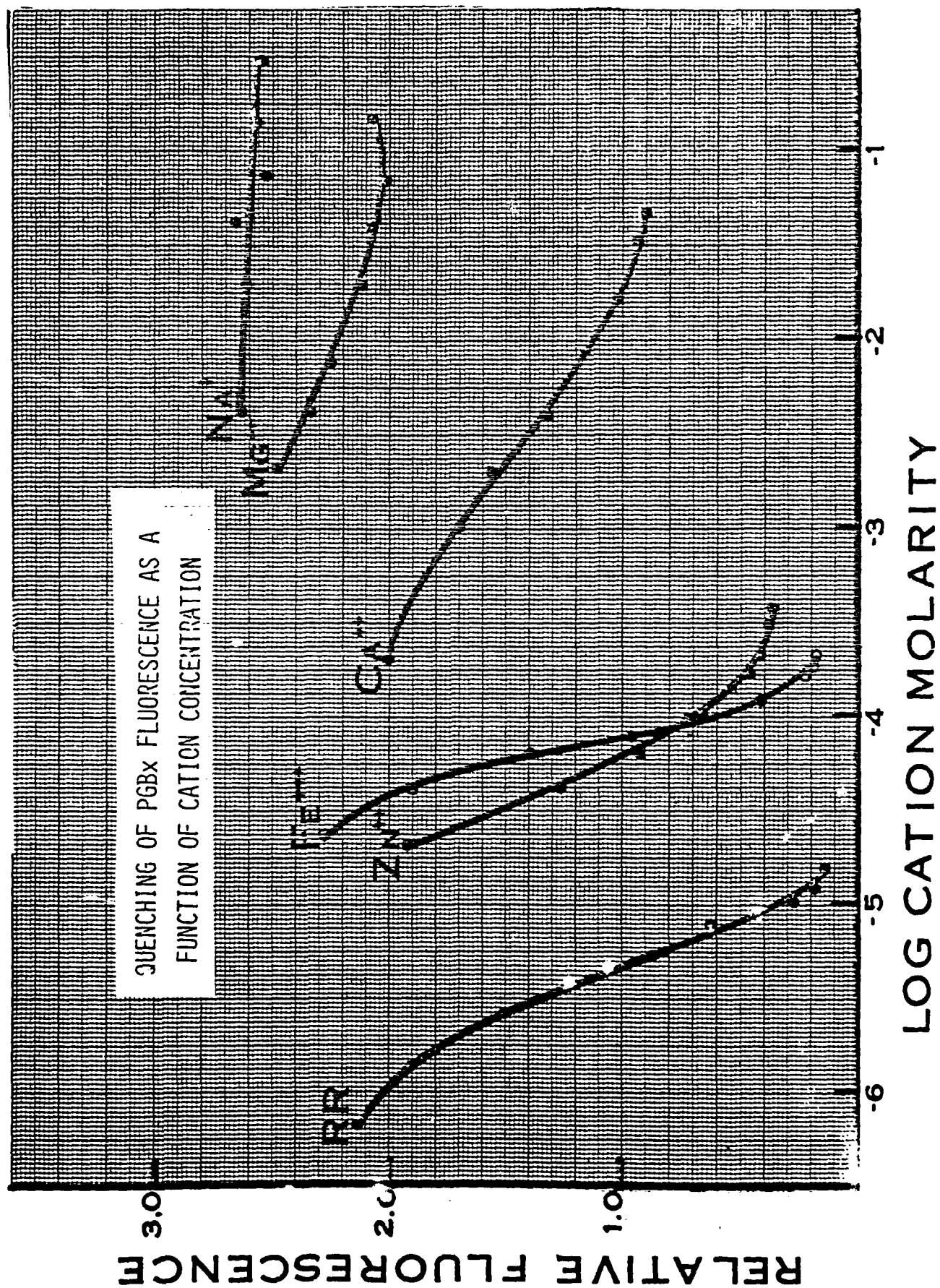SAMPLE PGBx 1860-2
SAMPLE CONC 1 mg/mL AMT. IN. 20 µL 20 µg

FLOW   1.00
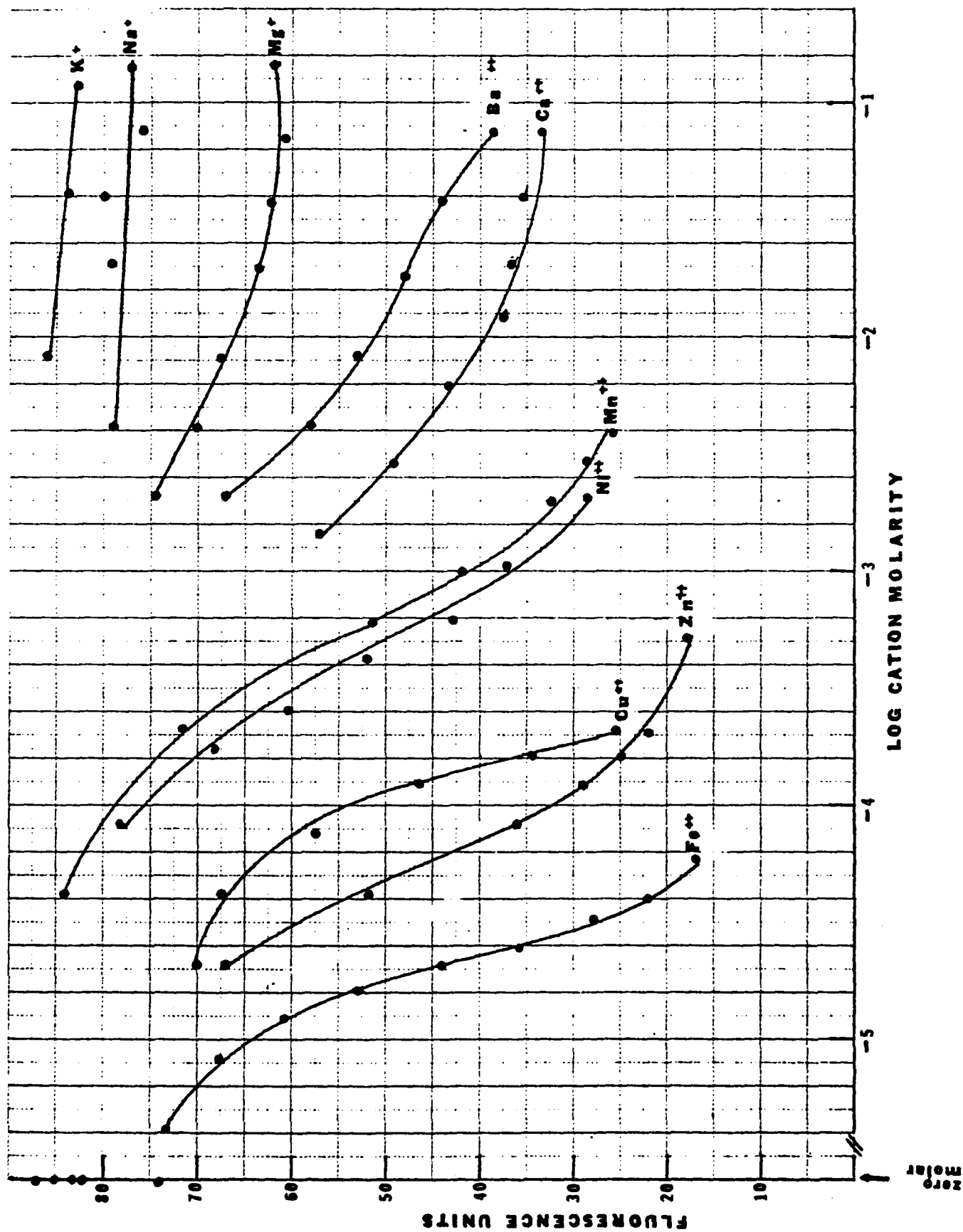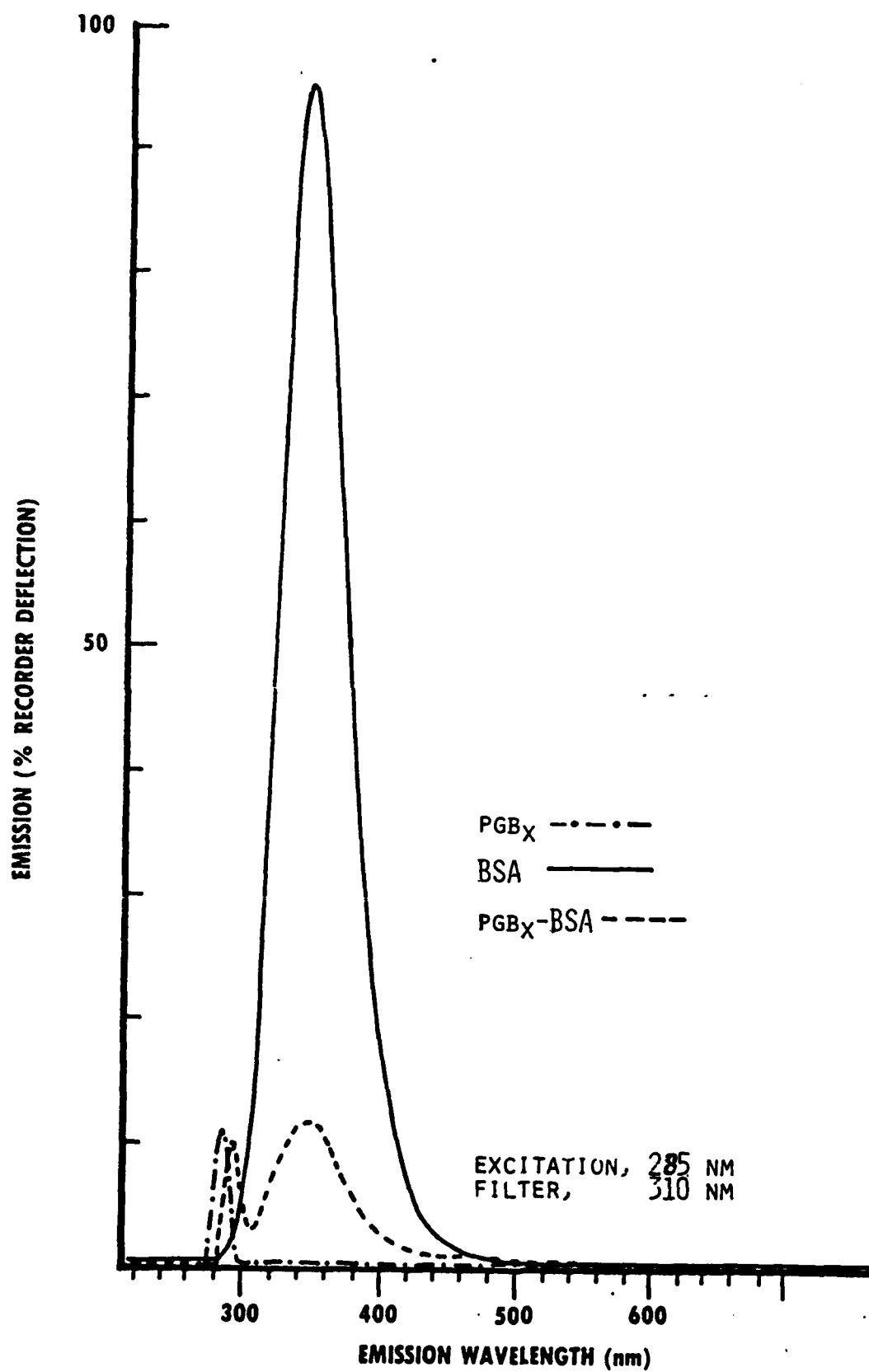%B   90.00   0.98
COLUMN'P.   91.1
MAX P   400   29
MIN P   0
S-TEMP A   40   40
S-TEMP B   40   40
OVEN TEMP   40   40
UV SGNL

CHT SPD   0.80
ATTN 2↑   6.0
ZERO   10.0
SLP SENS   0.10
AREA REJ   1000
OPTN   1

6.47
18.83
11
12.41
12.27

START
INJ

FIGURE 10

QUENCHING OF PGBx FLUORESCENCE AS A
FUNCTION OF CATION CONCENTRATION

LOG CATION MOLARITY

RELATIVE FLUORESCENCE

FIGURE 11

- 52 -

FIGURE 12

EXCITATION, 285 NM
FILTER, 310 NM

PGB_X ·—·—·—

BSA ————

PGB_X-BSA ————

EMISSION (% RECORDER DEFLECTION)

EMISSION WAVELENGTH (nm)

FIGURE 13

THE EFFECT OF PGB$_x$ ON THE EMISSION
INTENSITY OF BSA

EMISSION,   350NM
EXCITATION,   285NM
FILTER,   310NM

PGB$_x$ (μg/ml)

EMISSION (RECORDER DEFLECTION)
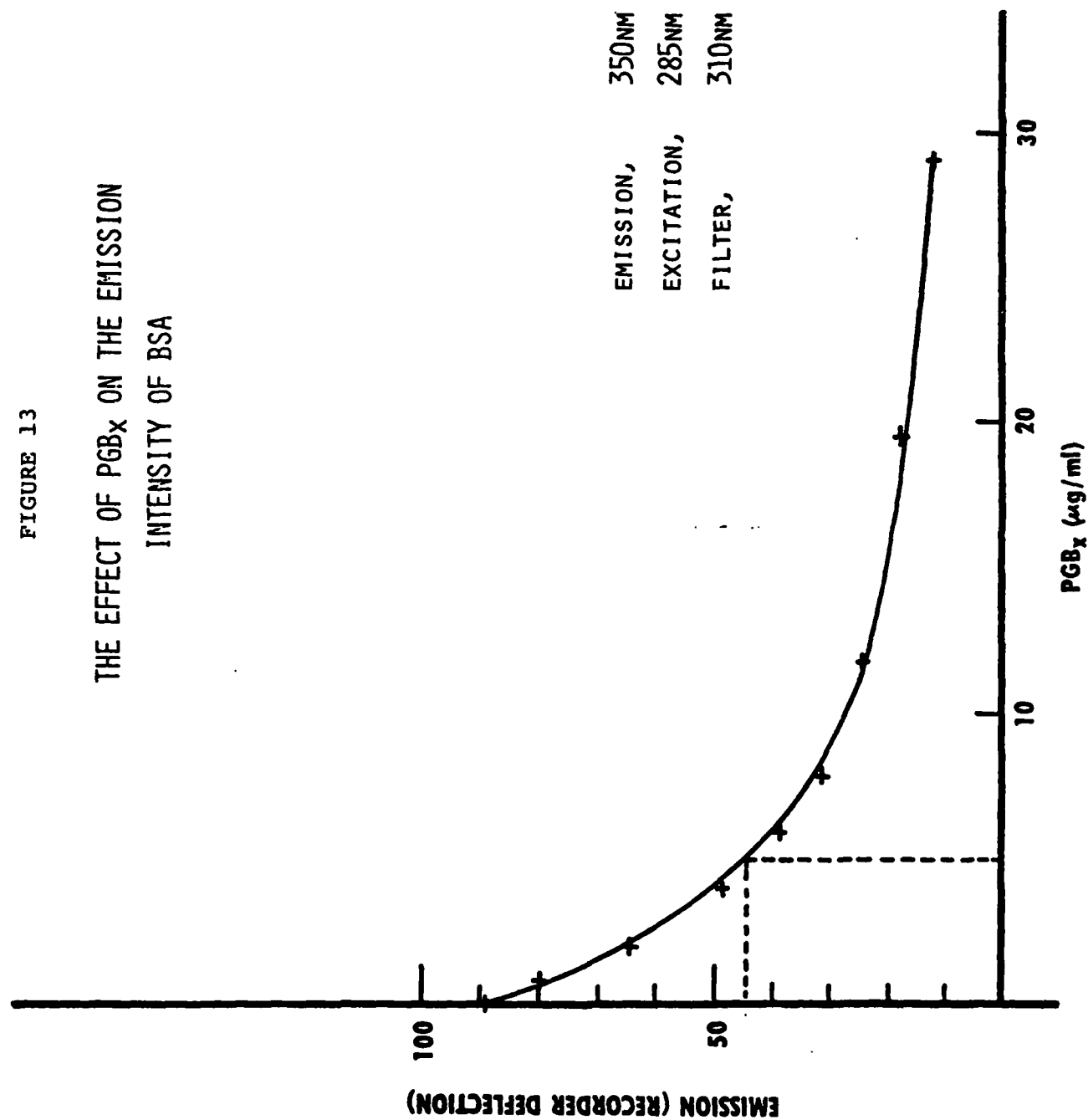
FIGURE 14

COMPLEX AS A FUNCTION OF PGBx CONCENTRATION
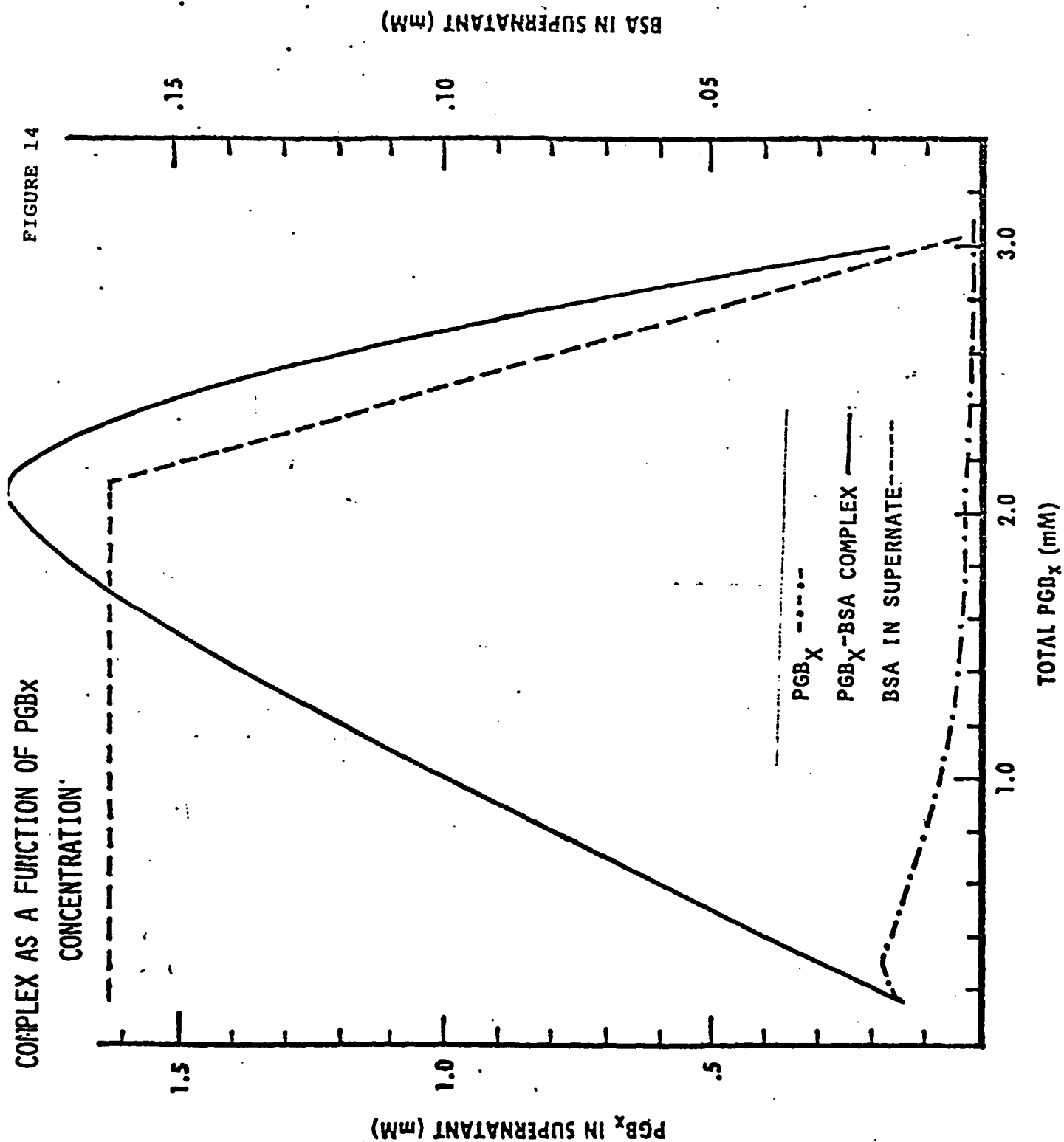
FIGURE 15

THE BINDING OF $^3H-PGB_{1.x}$ BY RLM

Pi ESTERIFIED

NO NUCLEOTIDE

Pi ESTERIFIED (mM)

$^3H-PGB_{1.x}$ BOUND (nMOLES/MG RLM)

46 1521

$^3H-PGB_{1.x}$ ADDED (mM)

FIGURE 16

46 1521

K·E 10 X 10 TO THE CENTIMETER   18 x 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

FR. 4
FR. 3
FR. 2
FR. 1

STANDARD PGBX

µMOLES P_i ESTERIFIED

FIGURE 17

PG-PX Activity Curves of
PG Bilayer Fractions 5, 6, 7, 8

STANDARD PGBX

μMOLES P_i ESTERIFIED

μG PGRx / 2.2MI REACTION
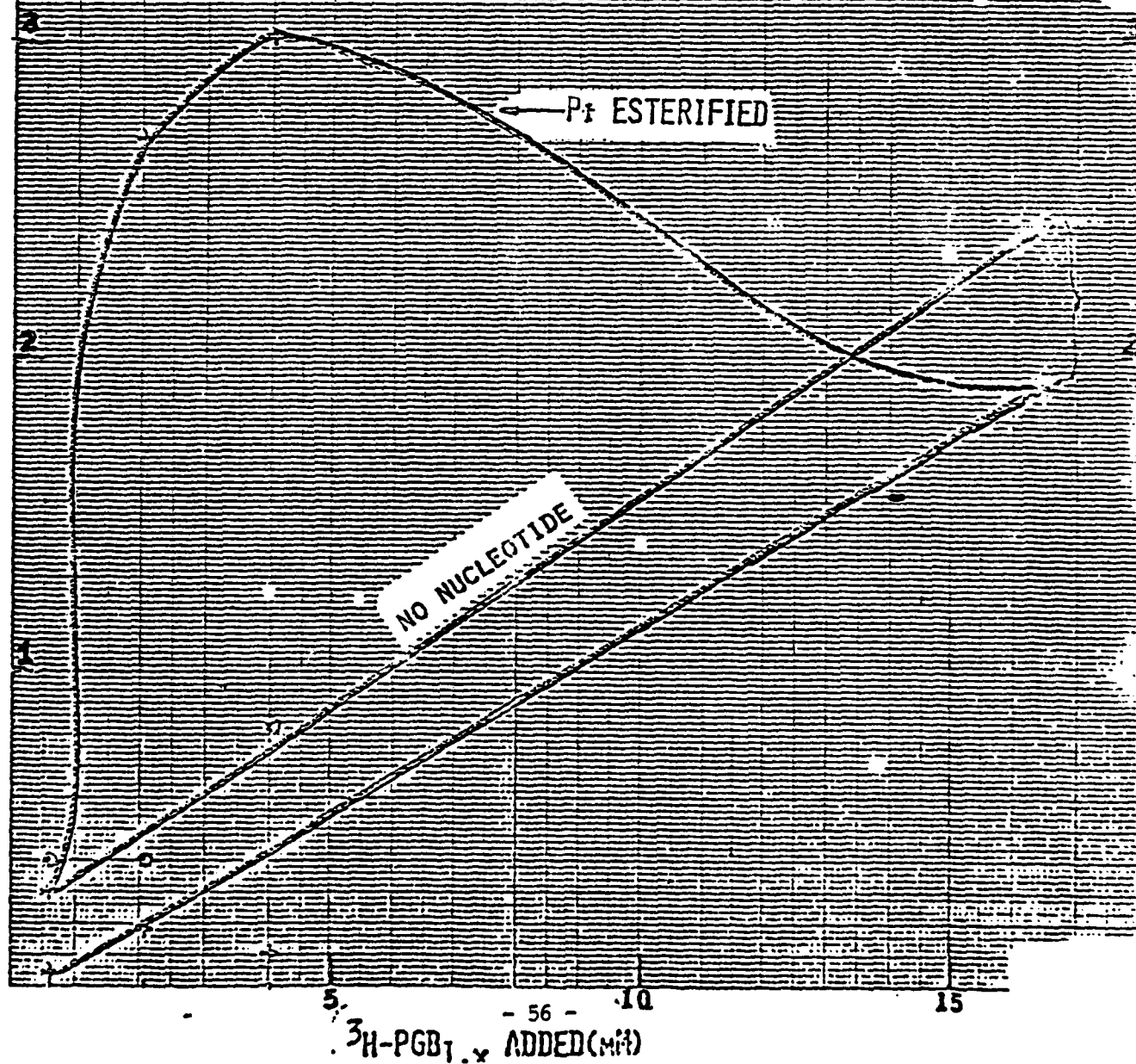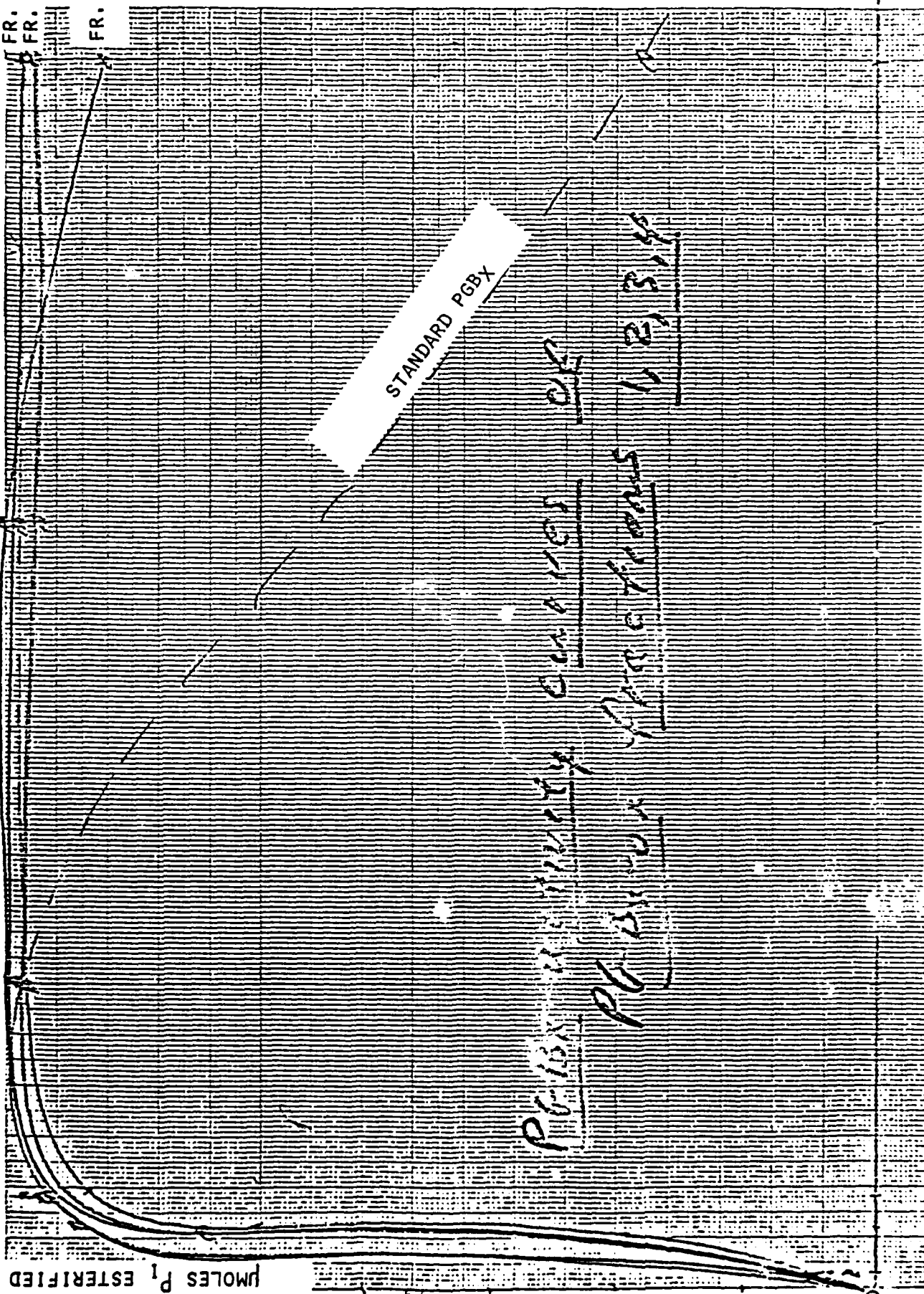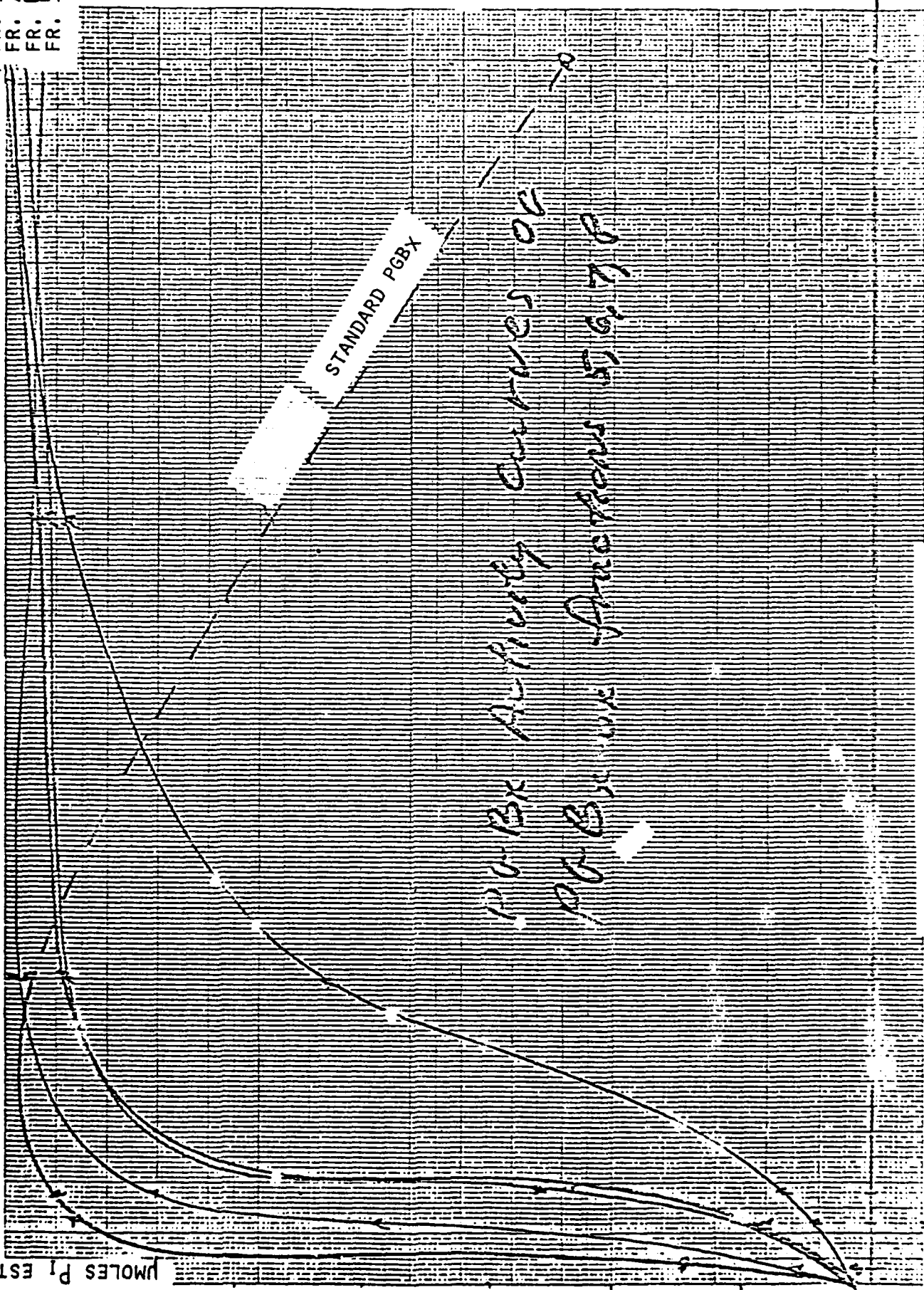
PART B   PHYSIOLOGICAL AND NMR STUDIES.

       1. PGB_x Enhancement of Survival of Hearts Exposed to Low

            Oxygen Tensions in the Intact Mouse

Introduction:

    Polis and collaborators showed that PGB_x prolongs the survival of

the heart in the monkey[1] and prolongs the survival of the brain in the

rabbit[2] when the blood supply is shut off. This was presumed to be due

to the beneficial effect of PGB_x on mitochondrial damage occurring[1,3,4]

during the period of ischemia. Experimental studies of PGB_x with large

animals such as monkey and rabbit involves much expense, labor and con-

sumption of valuable PGB_x. An analogous type of experiment using a small

animal is therefore much to be desired.

    We have undertaken the development of an equivalent experimental test

for a small animal, namely for the mouse. We have measured survival of

the heart in the mouse by the survival of the electrocardiogram in the

mouse. In brief we used a mouse subjected to low oxygen concentration

in the surrounding air (7.5% O_2) compared to the normal 21% oxygen. We

measured the EKG of the mouse during this period of low oxygen tension.

    It was observed by other investigators[5] with large animals that the

effect of low oxygen tension was first to make the heart beat faster but

then after prolonged exposure to cause marked slowing of the heart which

was the onset of circulatory collapse. In mice under our conditions, we

did not see the initial increase in heart rate. The slowing of the heart

after prolonged exposure to low oxygen tension was very obvious with EKG

observations in the mouse. As exposure to low oxygen tension continued

still longer, the heart rate slowed progressively, usually accompanied

by a progressive decrease in the voltage (height) of the EKG, until finally the EKG disappeared completely. Under these conditions when the mouse was reexposed to normal oxygen tensions, the mouse never showed signs of life; neither by visual inspection of the mouse nor by return of the EKG. We therefore used the time of disappearance of the EKG as a criterion of death of the heart. We will not go into the question at this time as to whether the time of death of the mouse as a whole or other organs of the mouse, such as the brain necessarily had the same survival time. That is why I have called this study specifically a study of PGB_x effects on the survival of the _heart_ of the mouse.

Methods:

Adult normal male mice of K57BL/6J strain from Jackson Lab weighing 22 to 28 g were used. Oxygen deprivation was accomplished by placing the mouse within a closed chamber measuring about 9 inch cube which initially contained room air but into which pure nitrogen was released at a rate sufficient to lower the oxygen tension from 21% to approximately 7.5% within $1\frac{1}{2}$ minutes. Thereafter oxygen tension was held at $7.5\% \pm \frac{1}{2}\%$ as monitored by a Taylor Paramagnetic Oxygen Analyzer. PGB_x was administered as the Na salt at a dose of 30 µg/g interperitoneally at 20 minutes before the onset of an oxygen deprivation experiment.

Control mice were from the same groups of mice with the same date of birth but did not receive PGB_x. EKGs were measured by a modification of a procedure developed previously[6] in this laboratory for measuring EKG of rats subjected to acceleration stress. In brief the mouse EKG was amplified by a low noise amplifier and was recorded on the face of

a storage oscilloscope from which it could be erased by the observer
after each measurement of the height and interval between pairs of
successive electric waves of the heart. End point of the experiment as
mentioned before, which gives cardiac survival time, was taken to be
the time at which the electrocardiogram was no longer visible on the
oscilloscope screen.

<u>Results and Discussion</u>:

A typical time course of heart rate of the mouse during exposure to
7.5% O_2 for a PGB_x mouse and for an untreated control mouse are shown
in Figure 1. The entire pattern of change is obviously delayed in the
PGB_x treated mouse, compared to the control mouse. When these experiments
were repeated on a set of 20 mice in which ten were treated with PGB_x and
remaining ten were untreated controls, this obvious improvement in the
survival of the heart at low oxygen tension due to PGB_x was consistently
evident. The difference may be demonstrated in a conspicuous and clear
fashion from measurements of the survival times of the hearts in these
two groups of mice which are summarized in Table 1. As you can see,
PGB_x caused a mean increase in survival time of the heart of the mouse
by 17.2 minutes which is an increase to 239% of the mean survival time
of the control hearts. This difference is statistically significant at
the 2% level of confidence by the student t-test.

Actually the PGB_x mice improved even more than indicated by large
effects shown in Table 1. The reason is that the three mice treated
with PGB_x which are shown in Table 1 to have survival times of 60 minutes,
actually had far longer survival times; in fact those three PGB_x treated

mice are still alive today, weeks after the experiment was concluded. At 60 minutes we got tired of watching the EKG and took these mice out alive. In the control group not even one mouse survived longer than 20 minutes. These 60 minute survival data are tabulated in Table 2. Therefore loċ̣ ̧ at the experiment this way it appears that not only did PGB_x lengthen the survival of the mouse heart but lengthened the survival of the mouse as a whole.

The difference in mouse heart survivals is not only statistically significant, it is also large enough to possess practical importance in physiology and medicine. It is consistent with large beneficial effects of PGB_x on ischemic hearts and brains of large animals observed previously by Dr. Polis and collaborators. The experiment on a mouse possesses the obvious additional virtue of ease and cheapness of performance. For the Navy, this result has obvious relevance, in that it offers a possible method to enhance survival of pilots at high altitudes.

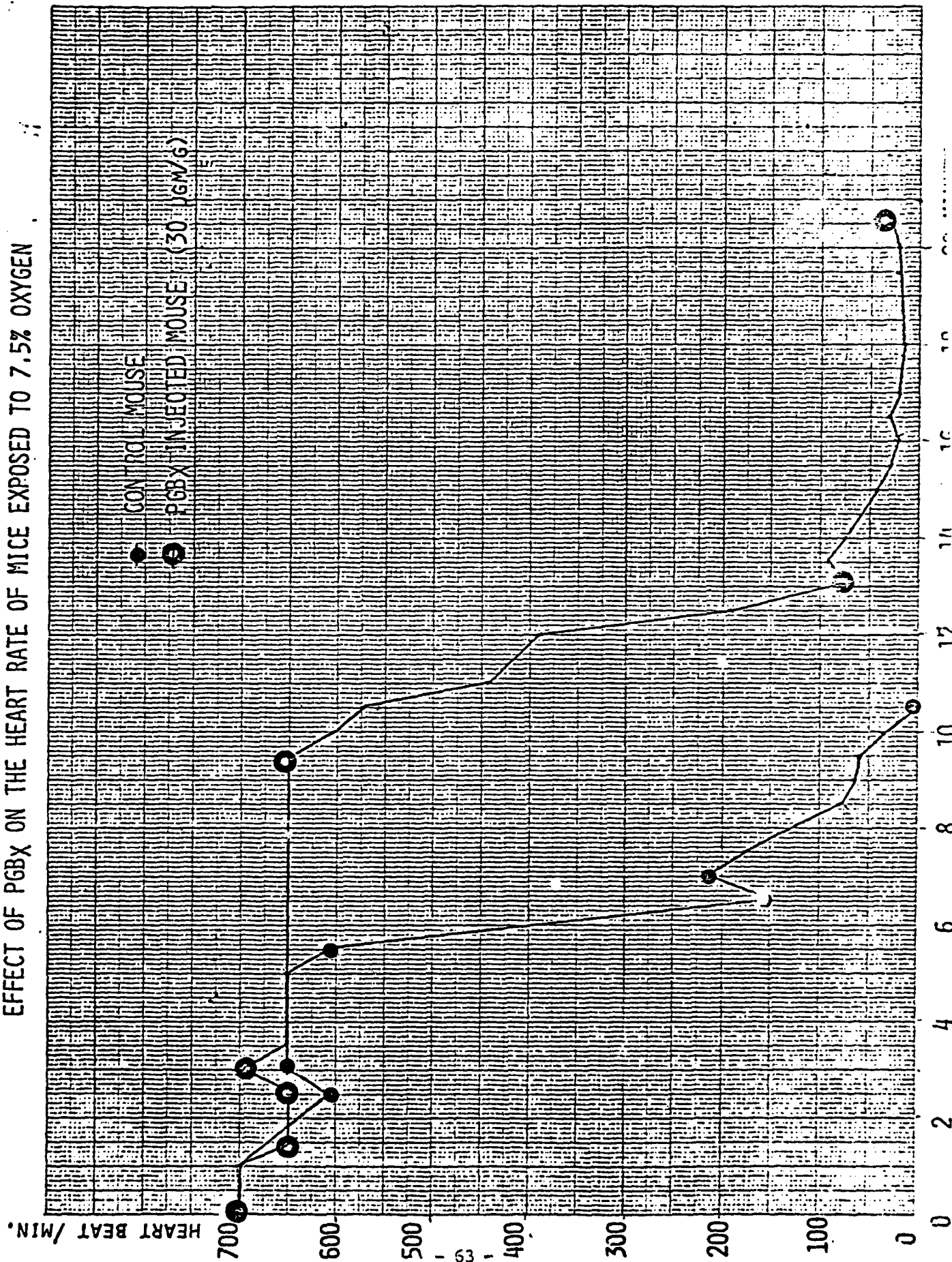EFFECT OF PGBx ON THE HEART RATE OF MICE EXPOSED TO 7.5% OXYGEN

TABLE 1

SURVIVAL TIMES OF MOUSE HEARTS

CONTROL MICE MINUTES	PGB$_X$ INJECTED MICE MINUTES
6.0	18.0
20.0	14.5
10.5	20.5
7.5	60.0
9.5	22.0
14.0	11.0
15.0	60.0
19.0	15.0
15.0	60.0
7.5	15.0

MEAN	12.4 MINUTES		MEAN	29.6 MINUTES

SE	1.56		SE	6.71
N	10		N	10

DIFFERENCE IN MEANS   17.2 MINUTES

% INCREASE IN SURVIVAL TIME
CAUSED BY PGB$_X$ +239%

STATISTICAL SIGNIFICANCE   2%

Table 2

Survivors after 60 minutes at 7.5% O_2

	Control	PGB$_x$
LIVING	0	3
DEAD	10	7
STATISTICAL SIGNIFICANCE		5%

2. Dissociation of Obesity Normalization and Glucose Effects of PGB_x in Mice

The diabetic syndrome of the hereditary diabetic mouse discovered by Jackson Laboratory was shown by us[7,8] to be normalized by PGB_x. The elevated blood glucoses, body weights and food intakes of this diabetic syndrome all are reduced toward normal by PGB_x, and the reductions are dose dependent[7,8]. It has been reported that this mouse diabetic syndrome does not respond to treatment with acute insulin administration, to food restriction, or to oral hypoglycemic drugs[9], and may be equivalent to the "adult" or insulin resistant form of human diabetes[10,11].

In the present study, we have sought to clarify the mechanism of the PGB_x action on the diabetic syndrome. We have been able to separate the PGB_x normalization of obesity and appetite from the PGB_x effect on blood glucose. We used a strain of mouse which is genetically different from, although closely related to, the diabetic mouse. This is the hereditary obese mouse[12], also supplied by Jackson Laboratory.

The hereditary obese mouse has a blood sugar that is close to normal[12]. Its body weight and appetite are excessively large[12]. The obese mouse resembles the diabetic mouse in that the blood insulin concentration is abnormally high[12], as described by Coleman. It has been surmised that in both obese and diabetic mice, there is a deficiency in the ability of the cells to use insulin[9,12]. The difference between the two types of mice

appears to be that the diabetic mouse is unable to maintain the high rates of insulin production necessary for adequate cell utilization of glucose and therefore eventually goes into pancreatic failure in its attempt to do so[9,12]. The obese mouse apparently has a stronger pancreas which is able to meet the abnormally high requirement for insulin production, and consequently is able to hold its blood glucose down close to a normal level despite its difficulty with the cellular use of insulin.

Results:

The first experiment (Fig. 2) using a single dose level (10 µg/g of PGB_x daily) established that PGB_x markedly reduced body weight gains of hereditary obese mice during growth. The second experiment (Fig. 3) confirmed this result, and in addition demonstrated that the normalization of body weight by PGB_x in obese mice was dependent upon the dose level of PGB_x. Bigger doses of PGB_x produced bigger reductions of body weights. These effects are statistically significant at the 0.1% level of confidence.

Only slight elevations of blood glucose were observed in the untreated obese mice. These were slightly but consistently reduced by PGB_x treatments (130 mg% for the untreated obese mouse to approximately 80 mg% of glucose for the PGB_x treated obese mouse).

Discussion:

These experiments have demonstrated that PGB_x can produce marked reductions of hereditary abnormalities of body weight and appetite with only minor effects on blood glucose. This would seem to indicate that the body weight and appetite effects of PGB_x are not mediated by way of a blood sugar mechanims.
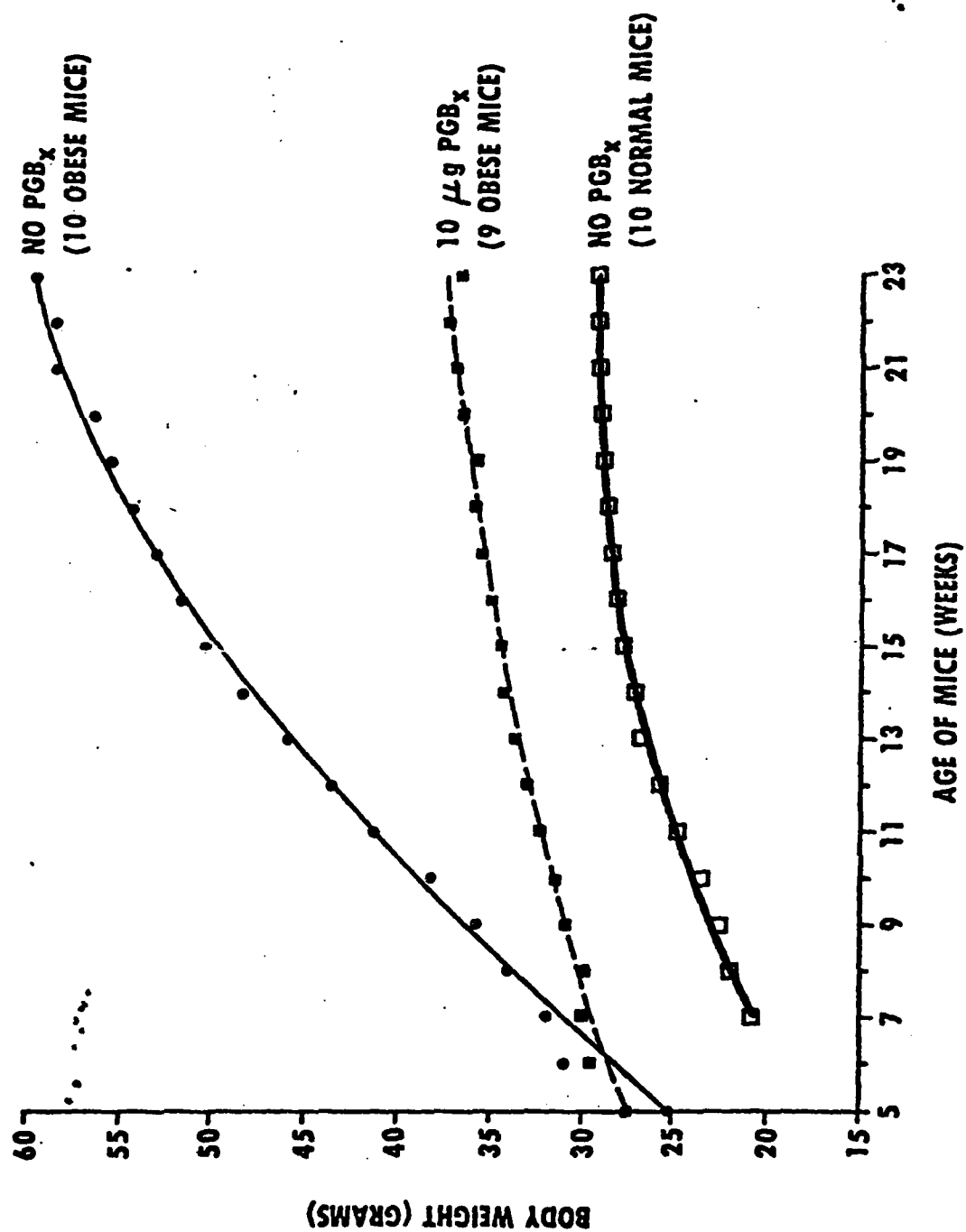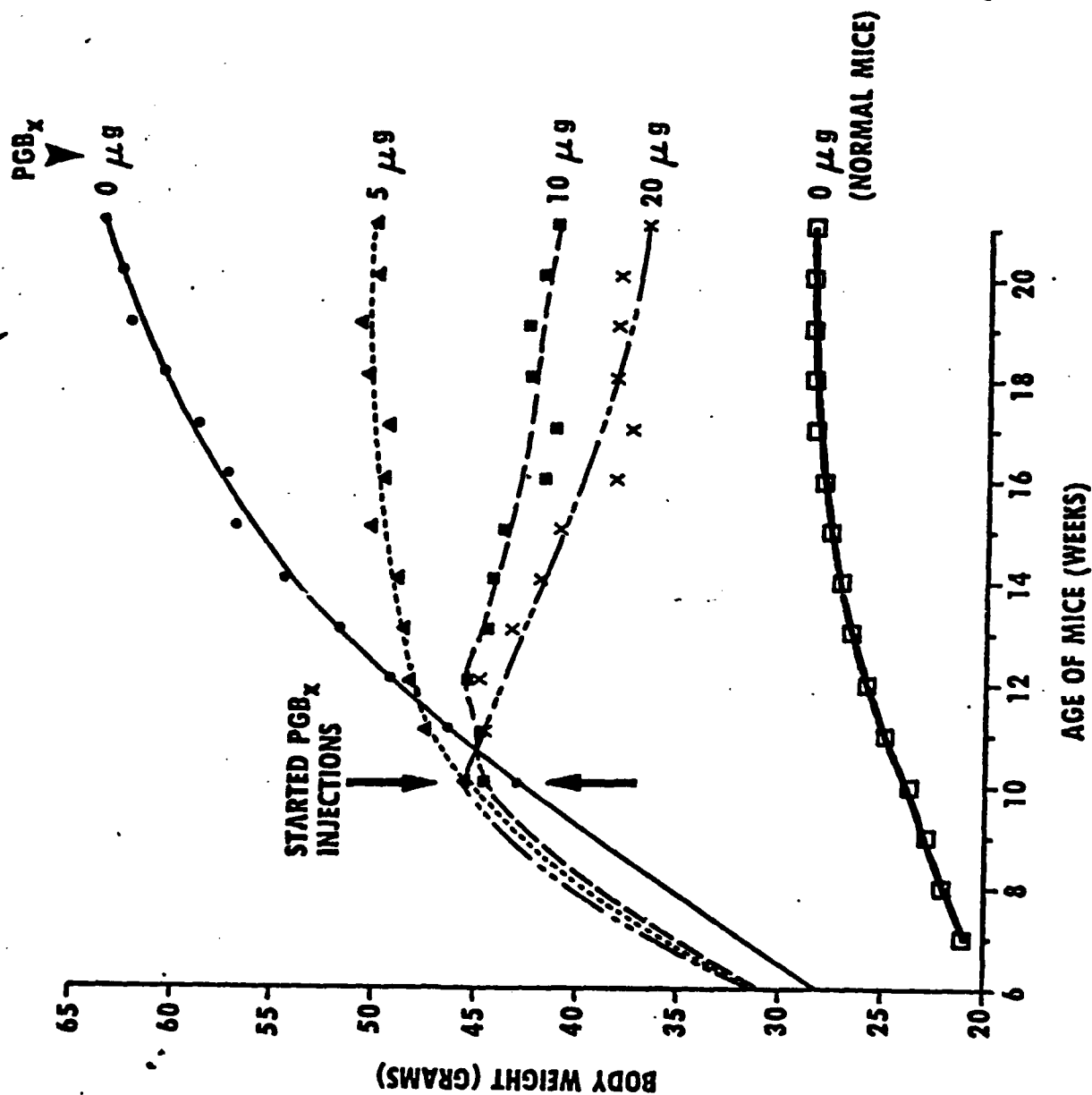
Fig. 2

OBESE MICE
PGB_x EFFECT ON BODY WEIGHTS

NO PGB_x
(10 OBESE MICE)

10 μg PGB_x
(9 OBESE MICE)

NO PGB_x
(10 NORMAL MICE)

BODY WEIGHT (GRAMS)

AGE OF MICE (WEEKS)

FIG 2.

OBESE MICE
PGB$_x$ DOSE – RESPONSE EFFECT ON BODY WEIGHTS

PGB$_x$

0 μg

5 μg

10 μg

20 μg

0 μg
(NORMAL MICE)

STARTED PGB$_x$ INJECTIONS

BODY WEIGHT (GRAMS)

AGE OF MICE (WEEKS)

3. Blood Leukocyte Effects of PGB_x in the Mouse

It is well known that some <u>monomeric</u> prostaglandin interact with leukocytes[13,14,15,16]. These interactions are highly complicated and related to the immune response of the animal. We considered the question of whether the <u>polymeric</u> prostaglandin PGB_x might also interact with leukocytes. We approached this possibility by conducting a study of the chronic effects of PGB_x on blood leukocytes in mice. We found that PGB_x when given in therapeutic doses does indeed change the counts of both lymphocytes and neutrophils in blood of normal mice, as well as in hereditary diabetic mice and in hereditary obese mice. This provides a possibility that white blood cell counts might be used as a clinical indicator of PGB_x therapeutic effectiveness and/or toxicity.

<u>Methods</u>:

For total white blood counts, blood was obtained by stabbing the tail vein of the mouse and was collected in a heparinized micropipet. Total white blood counts were done by two methods; sometimes by visual microscopic counting and sometimes by electronic counting, both of which used standard clinical methods. Differential leukocyte counts were made from blood smears on standard glass slides, air dried and stained with Wright Stain. Percentages of lymphocytes, neutrophils and monocytes in the smears were determined from microscopic counts of 100 cells for each blood sample. PGB_x was injected daily (five times a week) intra-muscularly for a number of weeks as detailed in each table.

A different PGB_x preparation, PGB_x dialysate, was prepared from PGB_x by dialysis against pH 6.5 phosphate buffer. The dialysate showed in vitro activation of mitochondrial phosphorylation with less inhibition of phosphorylation at high PGB_x concentrations. Injections of the Na salt of the dialysate were given as described for the usual preparation of PGB_x.

PGB_1 monomer in the acid form was first dissolved in a small volume of ethanol (10mg PGB_1 in 1 ml ethanol) and was then quickly diluted with Na_2CO_3 in saline (20mg/100ml) at $40°$. PGB_1 concentrations of 5mg/ml were used for injections which were given daily (five times per week) intra muscularly.

Results:

Table 3 shows blood lymphocyte and neutrophil counts measured in normal mice untreated or injected with PGB_x. We may note that the counts observed by us in the first line of Table 3 are of comparable magnitude to those reported previously by other investigators[17]. It should be remarked that we found high lymphocyte to neutrophil ratios in the mouse in contrast to the opposite pattern which is observed in humans. Table 3 shows in addition that this dose of PGB_x, which is a therapeutic dose, increases both lymphocyte and neutrophil counts by more than 100%. These increases are statistically significant.

We then proceeded to determine whether the PGB_x effect observed above in the normal mouse might also be observed in abnormal mice. We measured blood leukocytes in hereditary diabetic mice and in hereditary obese but non-diabetic mice. The data in Table 4 show that in the absence of PGB_x

treatment, hereditary diabetic mice and hereditary obese but non-diabetic mice have counts that are in some cases different from the counts of normal mice. When PGB_x is administered (Table 5) to these two types of abnormal mice, marked increases in both blood lymphocyte and neutrophil counts are observed consistent with the effects of PGB_x previously observed in normal mice. These increases due to PGB_x are again statistically significant (Table 5).

These observed PGB_x effects on leukocytes incidentally provided us with an opportunity to determine whether our standard PGB_x preparation might possibly be different in this physiological respect from a new PGB_x dialysate preparation recently developed by us and also provided us the opportunity to determine whether PGB_x behaved in the living mouse like a prostaglandin monomer (PGB_1). We observed that the PGB_x dialysate acted like PGB_x in that it raised the <u>neutrophil</u> count markedly, but the dialysate was different from PGB_x in that it did <u>not</u> produce the increase in <u>lymphocyte</u> count which PGB_x induced. Monomeric PGB_1 differed from polymeric PGB_x in that PGB_1 failed to produce the large increases induced by PGB_x in both lymphocyte and neutrophil counts (Table 6).

Dose-response curves for PGB_x on blood leukocyte counts were also performed. Similar dose-response patterns for PGB_x were seen in normal mice, in hereditary diabetic mice and in hereditary obese mice (Fig 4 and 5). For blood neutrophils, increases in the PGB_x dose from 0 to 20 µg/g progressively increased the neutrophil counts above the untreated animals. For blood lymphocytes, with PGB_x doses up to 10 µg/g the lymphocyte count was increased, but further increase in dose of PGB_x caused the count to decline (Fig 4 and 5).

Since our control mice in this series of experiments were
not injected, as a control on our technique, we injected a group
of normal mice with isotonic saline daily (5 days per week)
for 7 weeks and analyzed the blood for total white counts and
differential leukocyte counts as described before (Table 7).
The results of Table 7 showed that there were no significant
differences in the leukocyte counts between saline injected
and uninjected normal mice.

Discussion:

PGB_x resembles other known prostaglandins in that it has
significant effects on leukocytes. There appears to be a
fundamental change in the nature of the PGB_x effect for doses
higher than $10\mu g/g$, as manifested by the reversal of direction
of the lymphocyte effect. The lymphocyte count might possibly
be useful as a indicator for the onset of toxic side effects.

Table 3

PGB$_x$ Effect on Blood Leukocytes of Normal Mice

Mice	Treatment	Lymphocytes[a]	Neutrophils[a]
Normal	Untreated	11,340	1,380
Normal	PGB$_x$ 10µg/g	24,800[b]	3,240[b]
% change due to PGB$_x$		+119%	+135%

a - Counts are mean values

b - Value is significantly different at the 0.1% level by Student's t-test from that for untreated normal mice

Starting at 6 weeks of age, 10 normal ♀ mice were given daily injections (5 days per week) of PGB$_x$ 10 µg/g body weight. Ten control mice were not injected. After 8½ weeks of PGB$_x$ injections, blood was sampled in both groups for white blood cells and differentials.

Table 4

Blood Leukocyte Pathology in Untreated Diabetic and Obese Mice

Type of Mouse	Lymphocytes[a]	Neutrophils[a]	No. of Mice
Normal	10,120	1,430	52
Diabetic	4,450[b]	1,850	35
Obese	8,520	1,580	15

a - Counts are in mean values

b - Value is significantly different at 0.1% level by Student's t-test from that of normal untreated mice

Normal untreated mice include ♂ and ♀ mice, ages 15 to 28 weeks at the time of blood sampling. Diabetic mice were all ♀ and were 15 to 25 weeks old at the time of blood sampling, while the obese mice were all ♂ and 16 to 23 weeks old at the time of blood sampling.

Table 5

PGB_x Effect on Blood Leukocytes in Diabetic and Obese Mice

		Lymphocytes[a]	Neutrophils[a]
Diabetic mice	untreated	5,340	2,040
	PGB_x 10µ/g	8,780[b]	3,730
% change due to PGB_x		+64%	+83%
Obese mice	untreated	7,090	1,330
	PGB_x 10µ/g	11,710[c]	2,900
% change due to PGB_x		+65%	+118%

a - Counts are mean values

b - Value is significantly different at 0.1% level by Student's t-test from that of untreated diabetic mice

c - Value is significantly different at the 5% level by Student's t-test from that of untreated obese mice

10 diabetic mice, ♀ of the C57BL/KsJ strain were 13 weeks old when PGB_x 10 µg/g was injected into the fat pad of the neck daily (5 days per week) for 15 weeks. The control group of 10 ♀ diabetic mice was not injected. At the end of the 15 week period blood was sampled for white cells and differentials as described in Methods. The obese mice all ♂ of the C57BL/6JOB strain, five mice per group. Five mice were injected with 10 µg/g PGB_x i.m. daily (5 times per week). Injections were started when mice were 5 weeks old continued for 12 weeks. The control group was not injected.

Table 6

Effect of Different Prostaglandin Preparations on
Blood Leukocytes in Diabetic Mice

Mice	Treatment	Lymphocytes[a]	Neutrophils[a]
Diabetic	untreated	4,730	2,100
Diabetic	PGBx 10µ/g	9,600	3,000
Diabetic	PGBx 10µ/g dialysate	4,940	3,580
Diabetic	PGB_1 10µ/g	2,790	2,130

a - Counts are mean values

The ♀ diabetic mice were 8 weeks old when injections were started which were given daily i.m. (5 times a week) for 7 weeks. Six mice were injected with purified PGB_x pH 6.5 Dialysate and six mice were injected with PGB_1 monomer. There were five mice in the uninjected control group. At the end of the 7 week injection period, white blood counts and differentials were determined. Ten PGB_x injected diabetic mice were treated for 8 weeks and were 13 weeks old when treatment was started.

Table 7

Effect of Isotonic Saline Injections on Blood Leukocytes of Normal Mice.

Mice	Treatment	Lymphocytes[a]	Neutrophils[a]
Normal	Untreated	9,876	1,264
Normal	Isotonic Saline	10,251	1,630

a – Counts are mean values

Starting at 7 weeks of age, 7 normal ♂ mice were injected daily (5 days a week) with 0.1 ml isotonic i.m. and 7 control mice were uninjected. After 7 weeks of saline injections, blood was sampled for white blood cells and differentials. Untreated and saline injected values are not significantly different by "student" t test for lymphocytes and neutrophyls.

<u>Figure Captions</u>

Fig. 4 - Blood lymphocyte and neutrophile counts in normal mice. PGB_x dose-response curve.

Fig. 5 - Blood lymphocyte and neutrophile counts in hereditary diabetic and in hereditary obese mice. PGB_x dose-response curve.

FIG. 4.

DOSE EFFECT OF PGBx ON BLOOD LEUKOCYTES IN NORMAL MICE
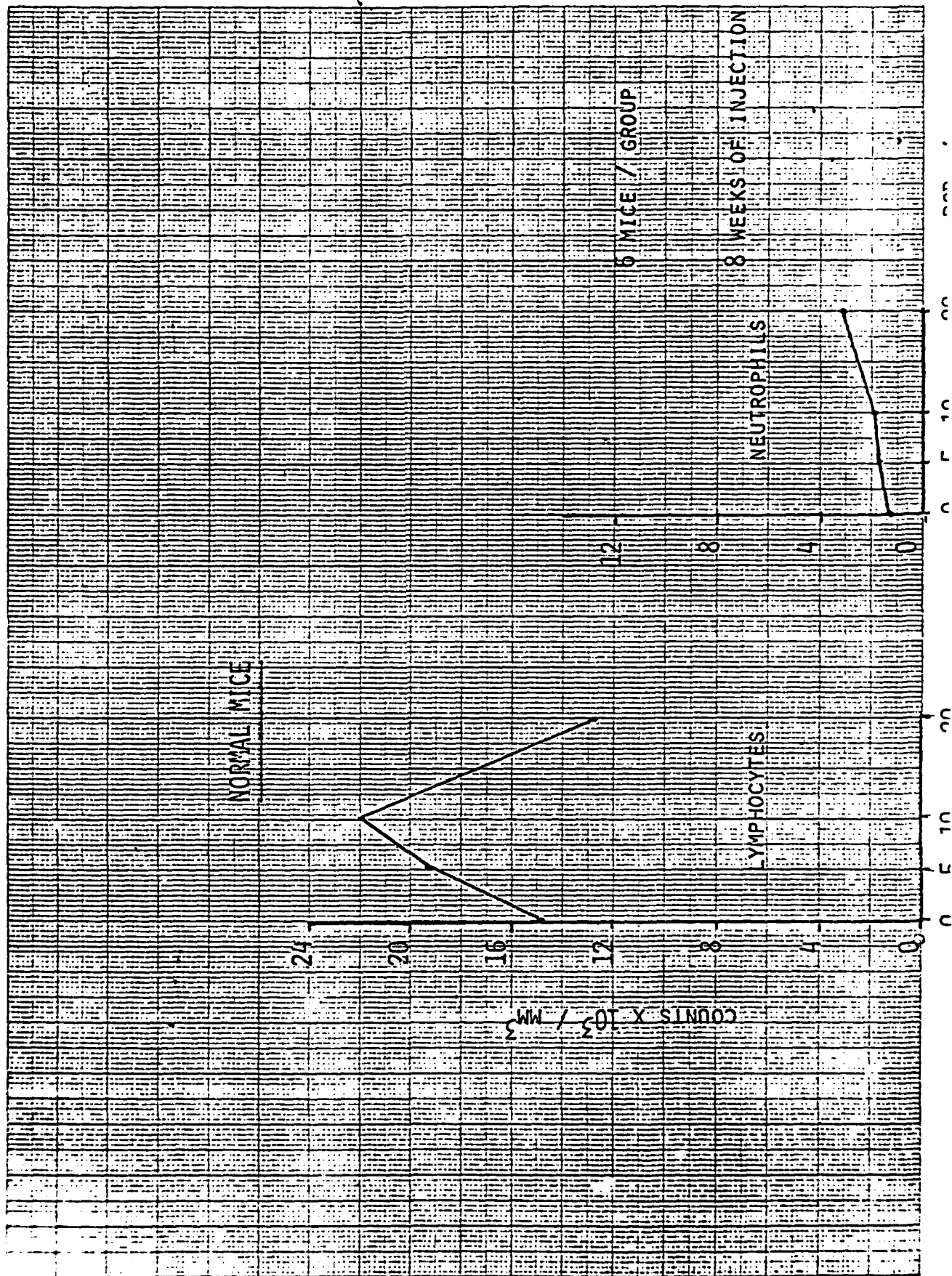
FIG. 5. DOSE EFFECT OF PGBx ON BLOOD LEUKOCYTES IN DIABETIC AND OBESE MICE

46 1521
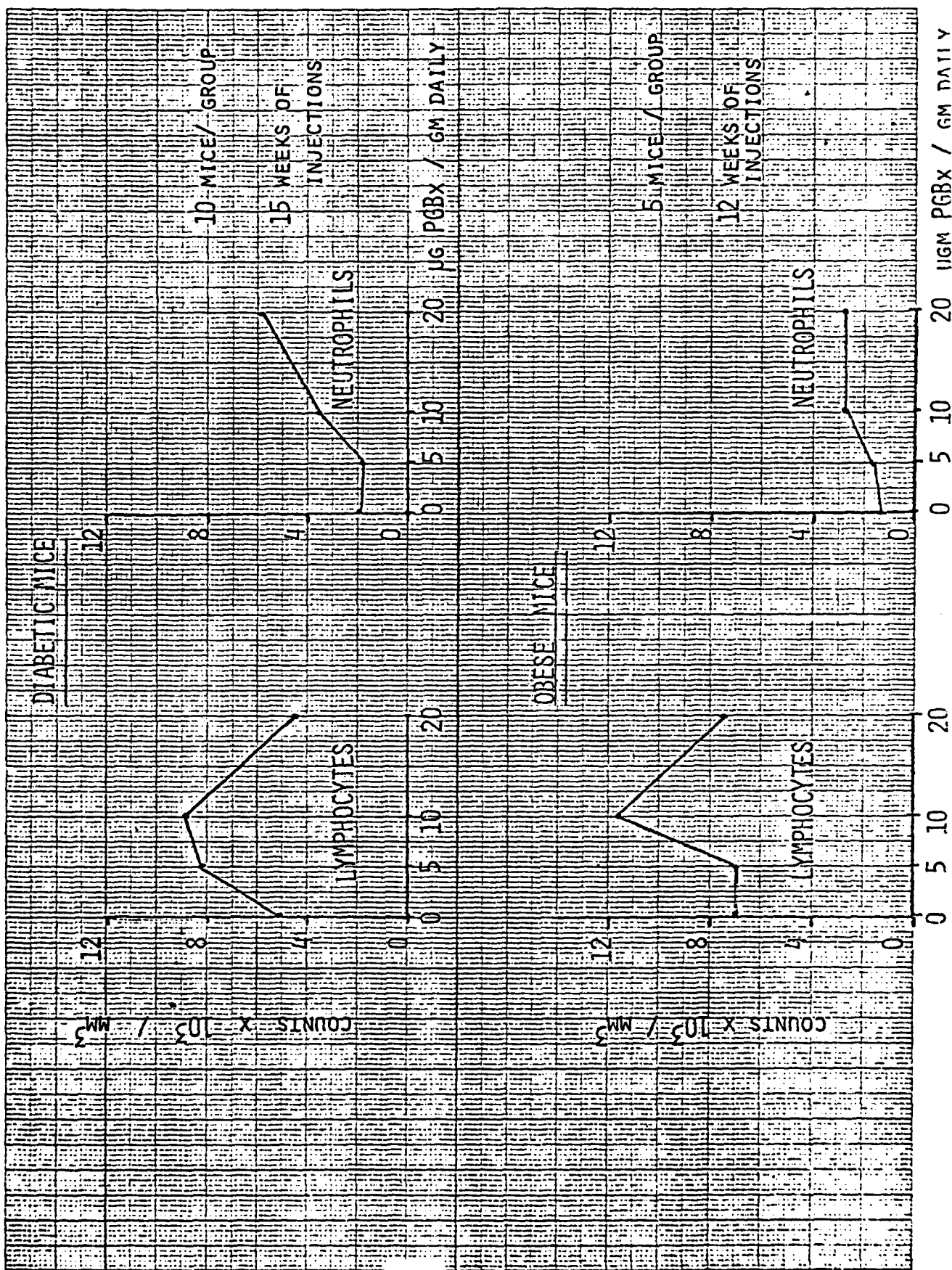
4. ^{13}C NMR Studies of Chemical Structure of PGB$_x$

We have done extensive ^{13}C NMR measurements of PGB$_x$, of its precursers and of possible analogs with the view toward establishing the chemical structure of PGB$_x$ in an exact and rigorous manner. We are not yet in a position to propose a complete structure of PGB$_x$. We are however in a position to propose the locations of some of the linkages between the monomeric units of PGB$_x$ and to make some surmises regarding the structures of the individual monomeric units.

On the basis of detailed studies involving the comparison of the PGB$_x$ spectrum with various smaller prostaglandin molecules of known spectra, we deduced that monomeric units of adjacent PGB$_x$ subunits may be joined by single bonds at C-16 to C-14. If this is so we might propose a partial structure of a PGB$_x$ hexamer as in Fig 6. This is certainly an incomplete proposal in that additional bonds probably link the monomeric units at other sites.

Our approach to the question of the linkages between the monomeric units of PGB$_x$ proceeded in detail in the following way: In Fig 7B' the ^{13}C resonances of PGB$_x$ in the carbonyl and double bond region are very similar to 15 keto-13, 14-dihydro-PGB$_1$ (Fig 7C) except that they are short and broad, and the areas under the curve integrate as 2 carbonyls, 1 carboxyl and 2 double bond carbons. When PGB$_x$ is compared to 15 keto-13, 14-dihydro-PGB$_1$ in the expanded $^{CH}2$ region (Fig 8 and 9) many PGB$_x$ carbon resonances although shortened and broadened are recognizable from

the monomer spectrum with the exception of C-16 and C-14, which have moved out of this region. This change is reminiscent of Dimer 3 (Fig 10, 10B). In the NMR spectrum of Dimer 3 the lines of some of the CH_2 carbons also have broadened and the resonances of C-16 and C-14 have moved out of the CH_2 region just as with PGB_x. These C-16 and C-14 resonances of Dimer 3 are now located in the methine region between 45 and 55 ppm (Fig 11). The NMR spectrum of PGB_x indicates a similar linkage from C-16 to C-14 which can be seen as a broad hump in Fig 7B'. In PGB_x, the C-14 and C-16 resonances of the monomers have disappeared from the CH_2 region. Therefore, regardless of what may possibly occur in dimers other Dimer 3, <u>in PGB_x we know that a cross linkage between adjacent monomeric units occurs at C-14 and C-16</u>. Therefore, we may with confidence write a partial structure for PGB_x as in Fig 6.

An alternative and/or additional crosslinkage in the PGB_x molecule is suggested by comparison of the PGB_x spectrum with the NMR spectrum of Dimer 1 (Fig 12) which has a linkage from C-10 of the cyclopentenone ring of one monomeric unit to C-13 or C-14 of the second monomeric unit. The observed similarities of integrated values of the carbon peaks of the PGB_x and Dimer 1 spectra suggest attachment of a crosslinkage at C-10 in PGB_x. Therefore we may write a partial structure for a PGB_x hexamer as shown in Fig 13.

The proposed structures of PGB_x hexamers in Fig 6 and 13 even when considered together still do not completely define our PGB_x preparations. We still do not know the sequences and combinations in which these two types of crosslinkages may occur between all adjacent pairs of monomeric subunits. Additional types of crosslinkages may possibly also be present.
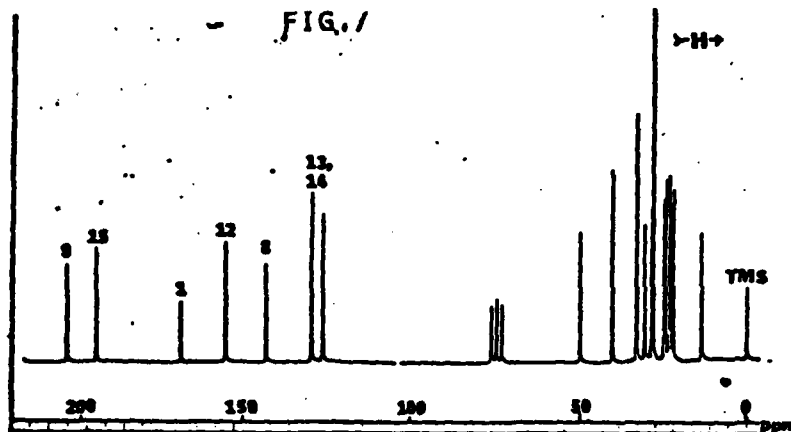
Fig. 6

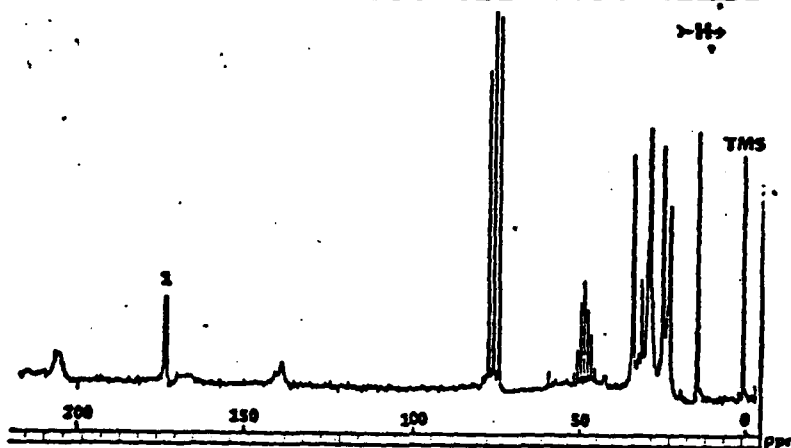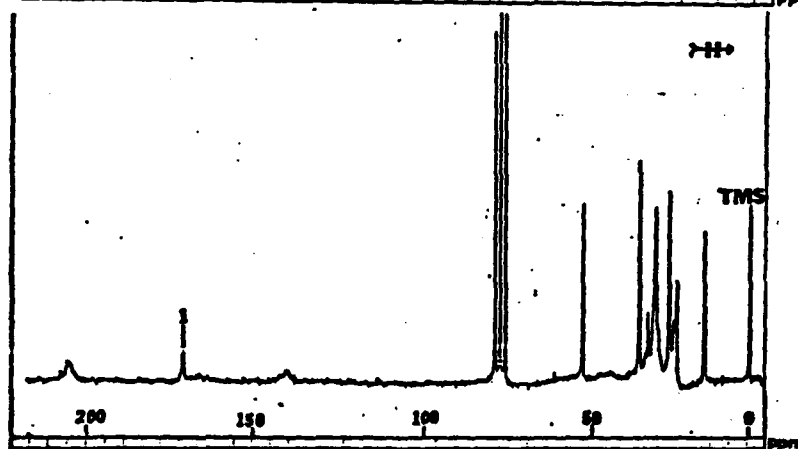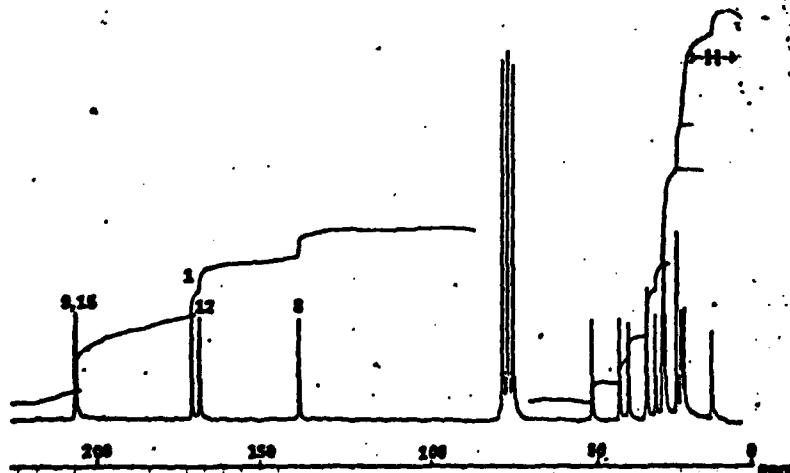PROPOSED PGBx HEXAMER WITH C-14 TO C-16 LINKAGE.

FIG. 1

C-13 NMR

A — 15 Keto PGB₁

B — PGBₓ

B' — methylated PGBₓ
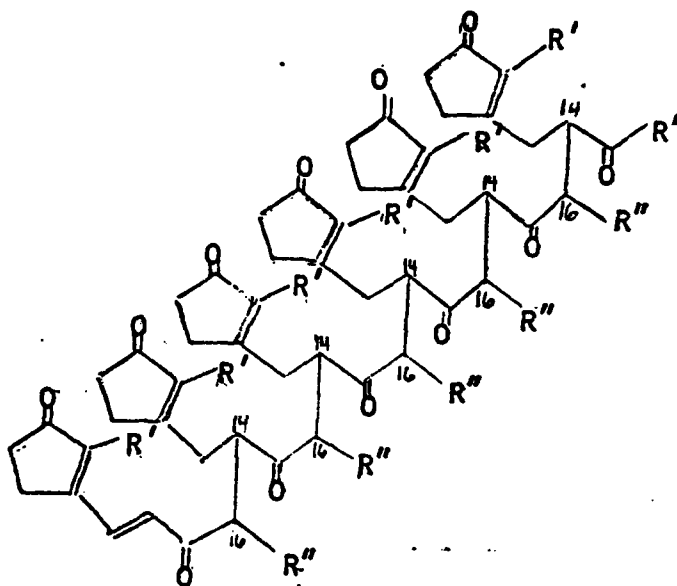
C — 13,14 dihydro 15 keto PGB₁

FIG. 8

CH2 REGION OF PGBx

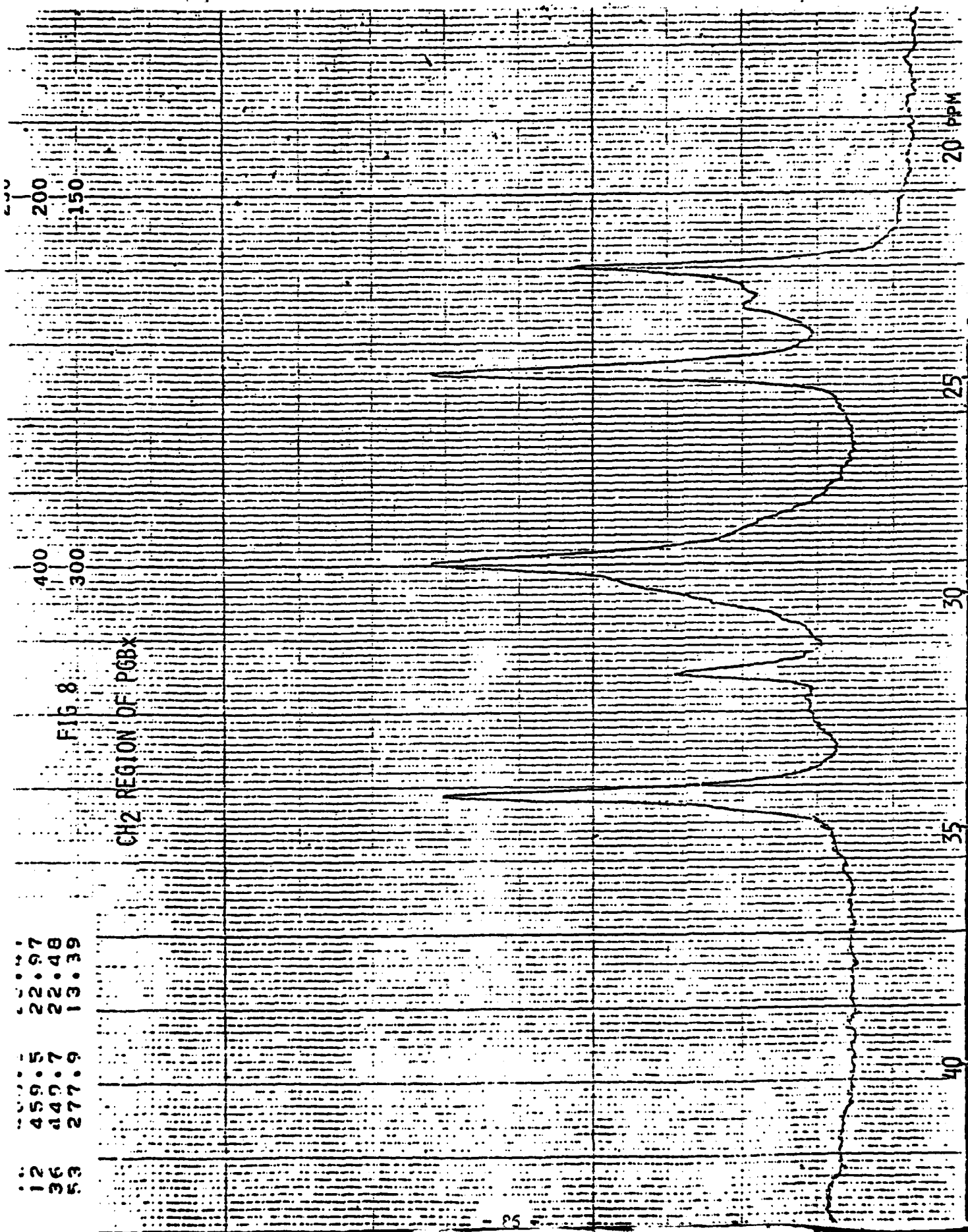FIG 9

CH₂ REGION OF 15-KETO-13,14-DIHYDRO-PGB1
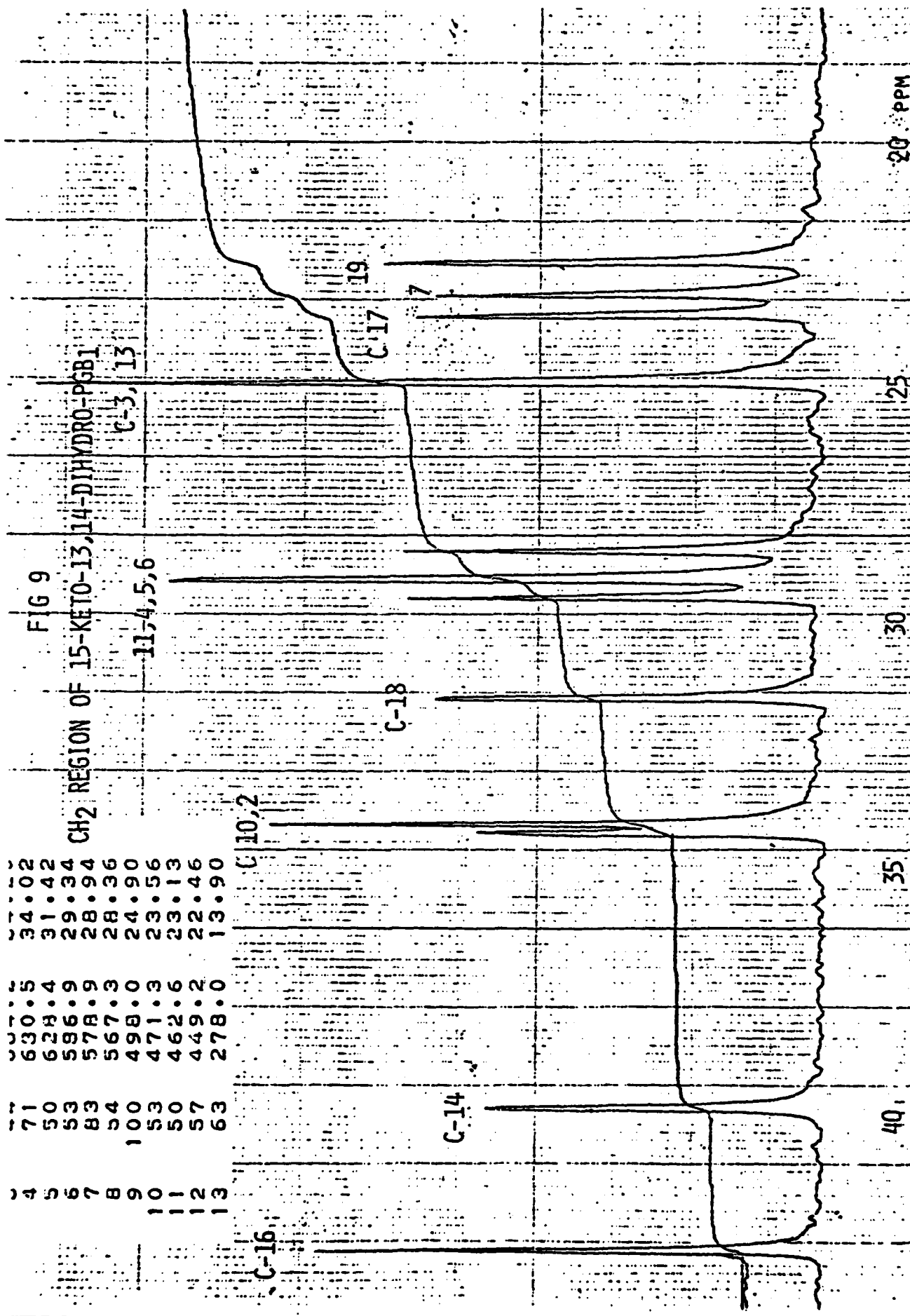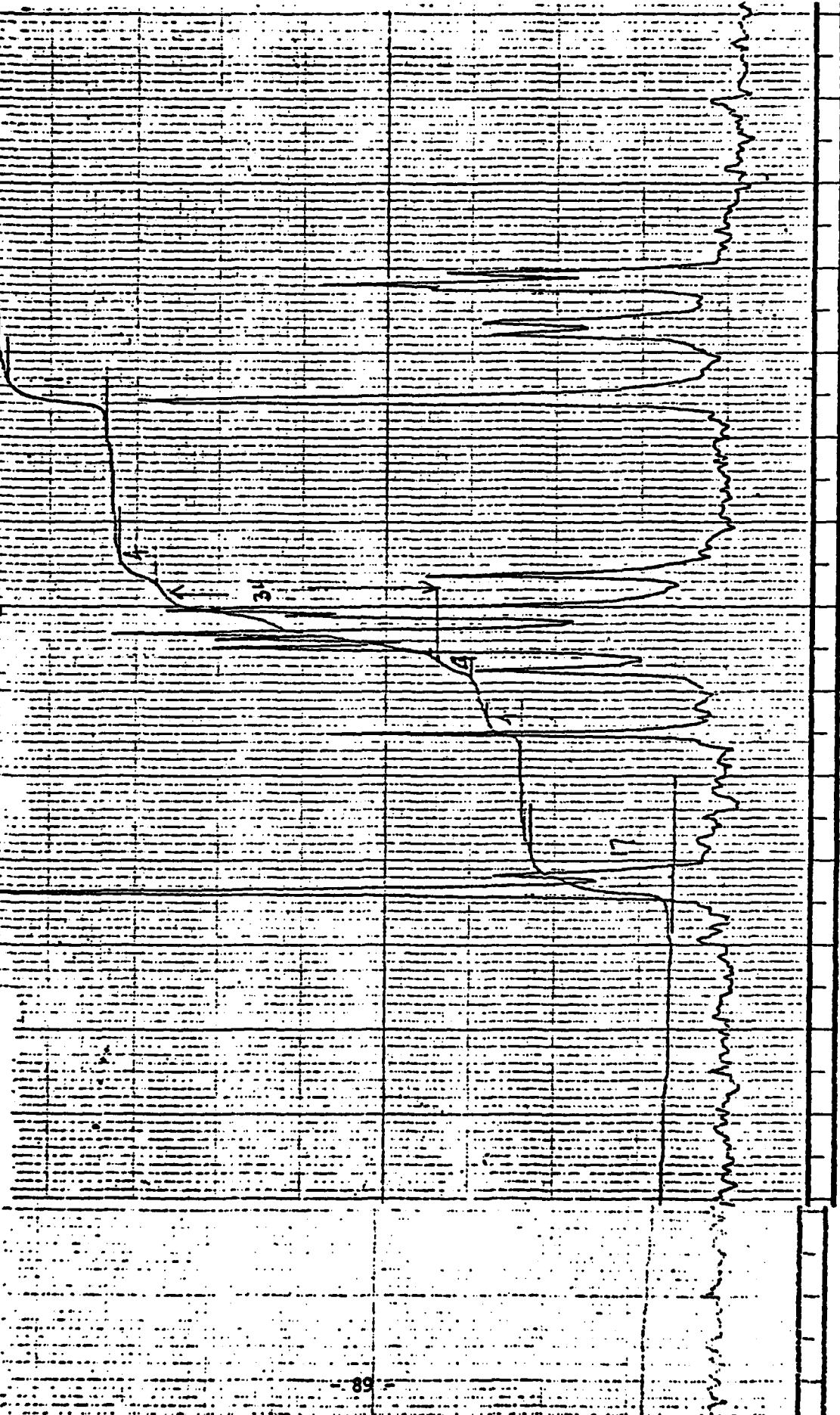
FIG 10

PROSTAGLANDIN DIMER 3
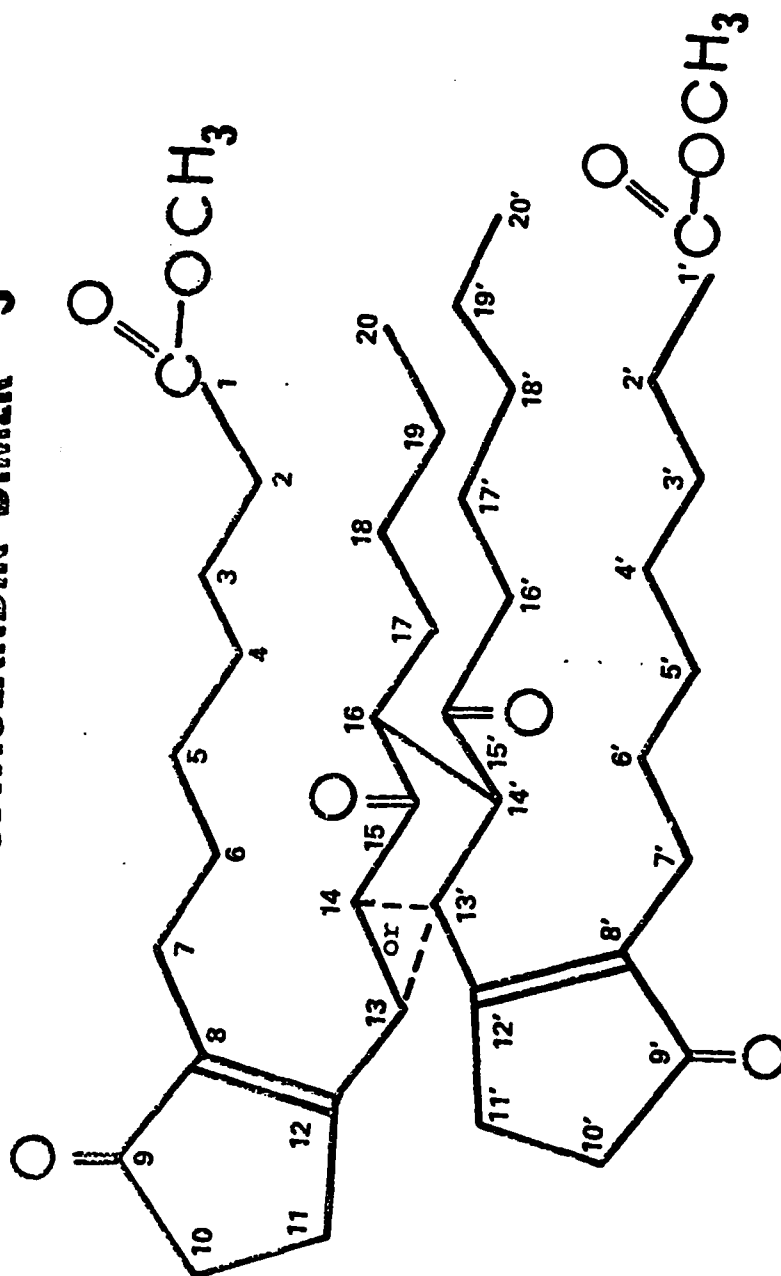
FIG 10B

DIMER 13 CH₂ REGION

30	457.1	23.35
36	453.8	22.68
48	452.1	22.60
34	449.0	22.39
54	235.0	14.25
66	276.1	13.90

3
4
5
6
7
8

34.x 31.76 29.97 28.71 24.82 23.58 22.68
      29.53 28.80   23.55 22.60
      29.36 28.15   22.39

FIG 11

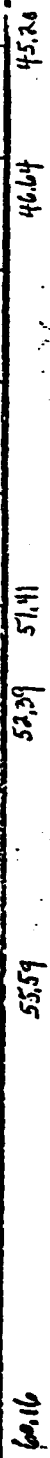DIMER 3 BRIDGE REGION
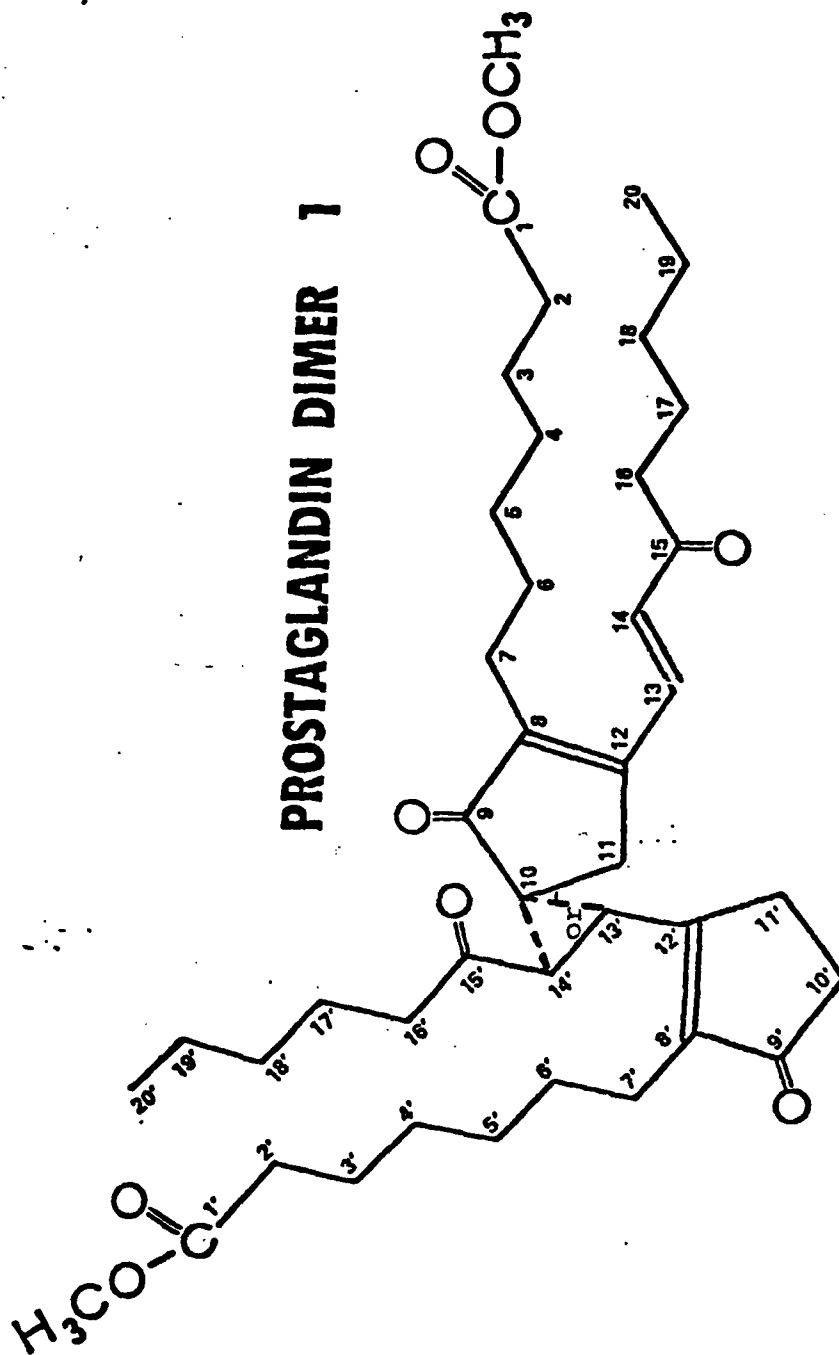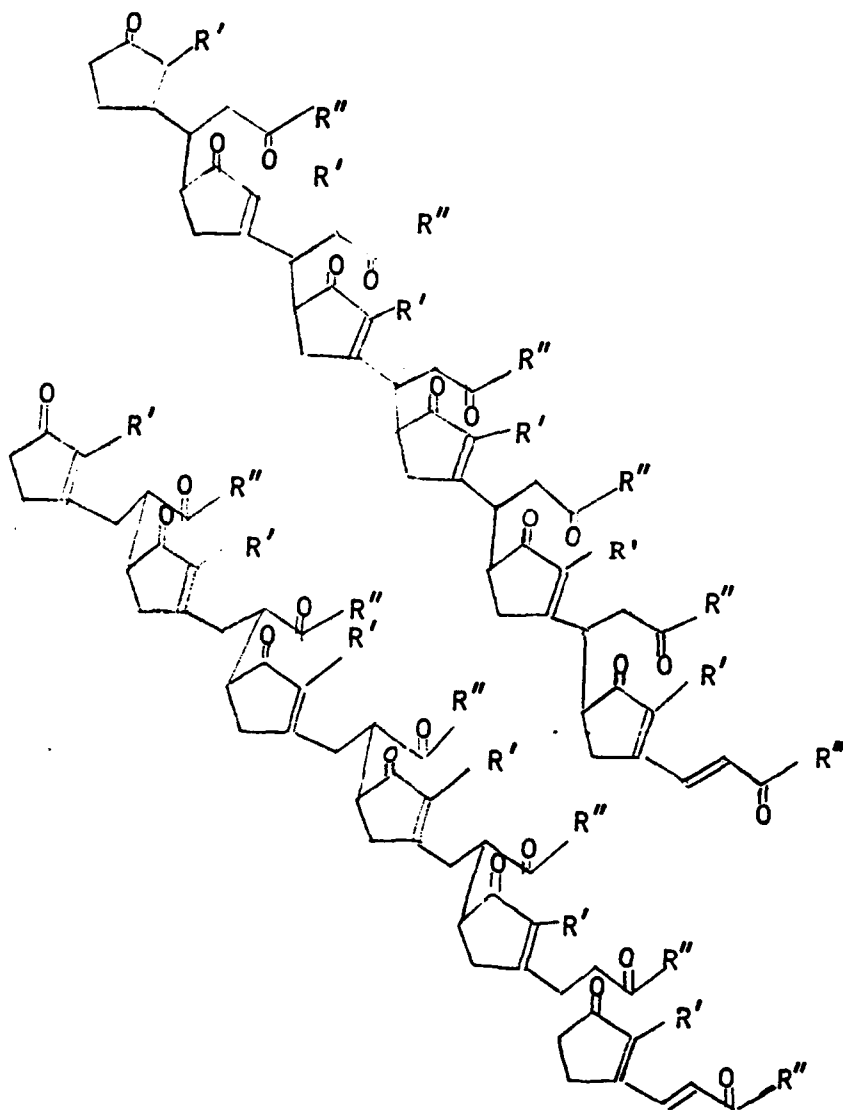
- 90

FIG 12

PROSTAGLANDIN DIMER 1

FIG 13

PROPOSED PGBx HEXAMER WITH LINKAGE ON THE CYCLOPENTENONE RING.

C-10 to C-13

C-10 to C=14

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	1.0		45		2.8		2.5
			50				
			56		3.2		2.2
			63		36		
			40				2.0
	1.1						
							1.8
	1.25		1.4		1.6		

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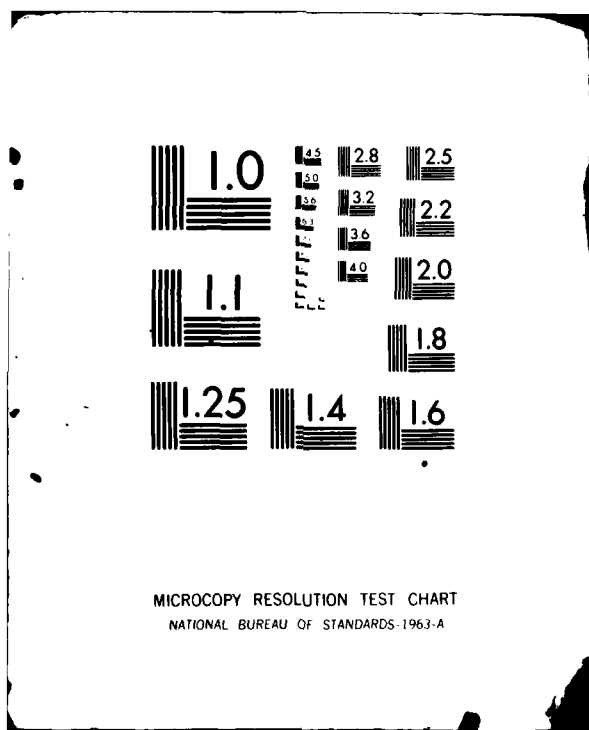
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